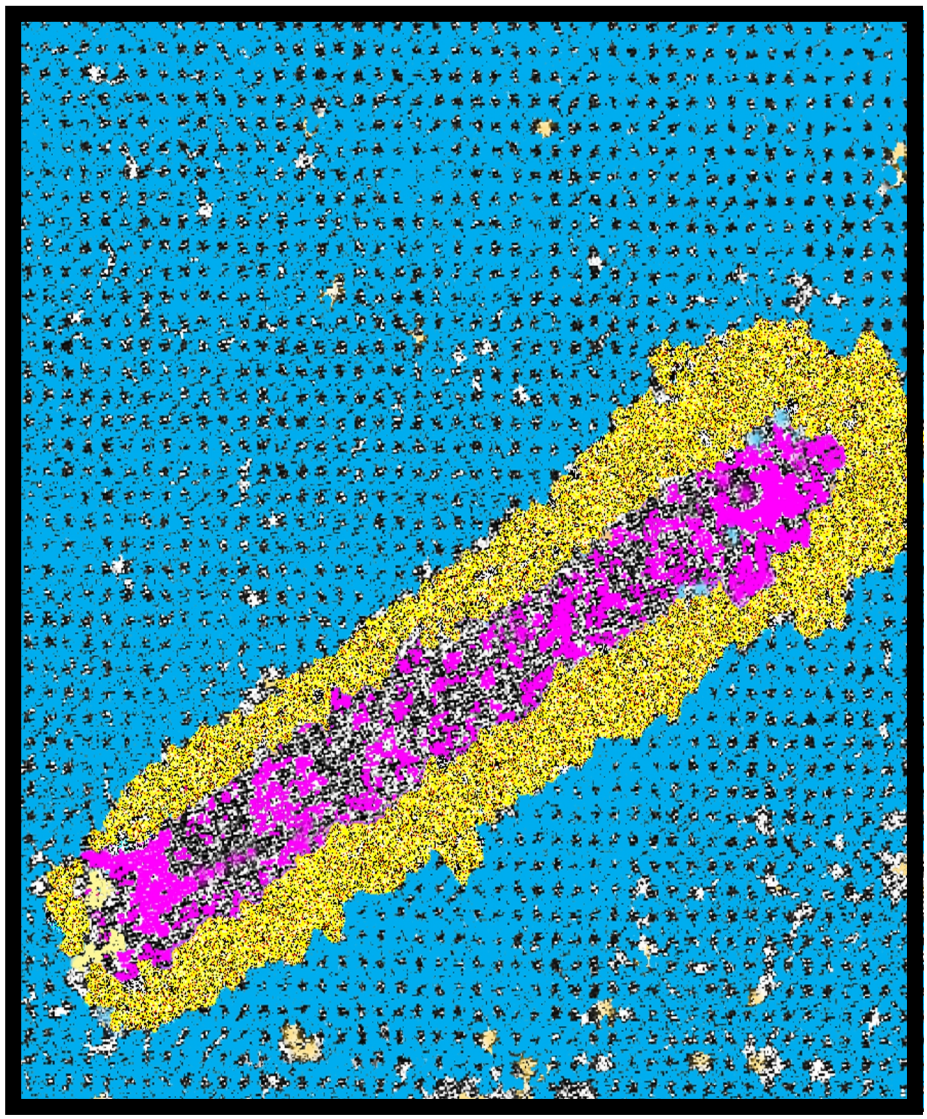
Baculovirus Molecular Biology

Second Edition



G.F. Rohrmann

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George F. Rohrmann

Department of Microbiology
Oregon State University
Corvallis, OR 97331-3804
rohrmanng@orst.edu

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Preface to the Second Edition

George Rohrmann, PhD.

Created: January 26, 2011.

Since the publication of the first edition of Baculovirus Molecular Biology two years ago, there have been significant advances in several areas of baculovirus research, most particularly in the characterization of gene function using bacmid technology. In addition, the long sought goal of determining the crystal structure polyhedrin was also recently accomplished. I have also added an additional chapter (Chapter 11) entitled 'Baculoviruses, retroviruses, and insect cells'. This is timely, not only because of my long standing interest in this relationship of baculoviruses and retroviruses, but also because of the significant advances that have occurred in the past two years on the cellular mechanisms by which endogenous retroelements are silenced in normal cells. I also found that many publishers allow online use of their figures without charge. This allowed me to include some classic pictures of baculoviruses along with more recent figures that will likely become classics. Because of the use of the *Bombyx mori* baculovirus for many investigations, I have now included the orthologous BmNPV orfs along with those of AcMNPV in the revised Chapter 12, 'The AcMNPV genome: Gene content, conservation, and function'. I am grateful to a number of individuals who contributed suggestions and comments for this revised book, including Drs. T. Ohkawa, S. Katsuma, L. Passarelli, A. Khan, and G. Pennock. This book could not have been produced without the continued encouragement and assistance of Dr. Laura Dean of the National Library of Medicine (NCBI/NIH).

Preface from first edition

Created: November 1, 2008.

After completing my PhD on an RNA bacteriophage in the Department of Microbiology at the University of Washington in 1970, I accepted a position as lecturer in biology at the University of Botswana, Lesotho, and Swaziland that was located in Roma, Lesotho. Because of pressure from the governments of Botswana and Swaziland for a presence of higher education in their countries, satellite two-year campuses were started in these countries in 1971, and I transferred to Swaziland to start the biology department. Swaziland has highlands on the west side that slope down to a more arid region to the east. One of the first things I noticed upon moving there was the diversity of the insect life compared to elsewhere that I had lived. I started photographing as many of the distinctive species that I could find, and this eventually led to an interest in research on insects.

I was given a three-month sabbatical by the university in the fall of 1974, and I spent it at Oregon State University because they had an entomology department and it was located near my family who lived in Eastern Washington. Because my training was in virology, I thought that a way to combine my past experience in virology with research on insects was to conduct research on insect virology. Adjacent to the university campus is a USDA Forest Service Forestry Sciences Laboratory that at the time had a very vigorous program in the biological control of forest insects, particularly of the Douglas-fir tussock moth, *Orgyia pseudotsugata*. The program in virology was led by Mauro Martignoni – who introduced me to baculoviruses -- and the *Bacillus thuringiensis* program was directed by Hank Thompson. There were also an electron microscopy lab staffed by Ken Hughes and Bo Addison and a variety of other individuals who did research on the formulation and application of biocontrol agents. During my brief time at OSU I started working on the characterization of baculovirus occlusion body proteins in the laboratory of Prof. Victor Brooks.

My interest in baculoviruses that began with my fascination with insects moved on to using viruses for biological control and eventually became focused on molecular biology. After I left OSU at the end of 1974, Mauro offered me a position as a lab technician. Therefore, I returned to OSU in the fall of 1975 and started my career in baculovirology. I initially worked in Mauro's lab, but because my research was directed toward developing methods of identifying baculoviruses, he encouraged me to work in the lab of Prof. George Beaudreau, a molecular biologist working with retroviruses in the Department of Agricultural Chemistry, who had collaborated with Mauro on a baculovirus project in the past. Another virologist, Prof. George Pearson, was located in the Department of Biochemistry and Biophysics that was in the same building as our lab. His group

was purifying restriction enzymes, and after a recent sabbatical in Prof. Phil Sharp's lab (whose group had recently discovered introns), he had begun investigating introns using electron microscopy. In addition, George Pearson's wife, Margot, an immunologist, worked in the Beaudreau lab and later worked with me for many years. Therefore, whereas my early interest was in insect biocontrol, due to the influence and opportunities provided by my association with these experienced molecular biologists, I initiated research on the molecular biology of the baculoviruses of *O. pseudotsugata* including restriction enzyme analyses, cloning, and intron analysis. Furthermore, I was always interested in evolution, and when we were able to obtain some N-terminal sequences for polyhedrin in collaboration with another colleague in the Department of Biochemistry and Biophysics, Prof. Bob Becker, George Beaudreau, and I set about attempting to examine the evolution of these proteins. Back in those days, the calculation of phylogenetic relationships was accomplished using hand calculators. Despite the limited data and primitive methods of analysis, these early data clearly suggested that baculoviruses had evolved with their host insects, and this concept has been verified with recent, more complete and extensive data sets using sophisticated computer programs.

The genesis for this book was my decision to completely annotate the AcMNPV genome in an attempt to understand what was known about the function of the ~150 open reading frames present. Although I found this somewhat challenging, with the assistance of the internet, particularly PubMed and the Viral Bioinformatics Resource Center (VBRC) at the University of Victoria in Canada, I was able to cover 10–15 orfs per day both by reviewing the literature and examining phylogenetic relationships using the VBRC site. Furthermore, Dr. Jun Gomi gave me permission to cite the work that she and her colleagues performed with Prof. Maeda on the deletion of all the genes of BmNPV. This was an important study, but because of the untimely passing of Prof. Maeda, much of this work has not been published. Because of the close relatedness of the BmNPV genome to that of AcMNPV, this information is invaluable in interpreting the function of many genes in AcMNPV that have not been characterized. Once I had compiled a draft of the information on AcMNPV orfs, I decided that since I had most of the references annotated, I would attempt to organize the material into a series of chapters that would cover baculovirus molecular biology, and in the process put many of the genes that I had annotated into the context of their role in the biology of these viruses. This idea was stimulated when I found that it would be possible to publish this book online with open access using Bookshelf, a scientific publishing arm of the National Center for Biotechnology Information of the National Library of Medicine (NLM). I thought that this was important because some of the most active labs in baculovirus molecular biology are located in areas where access to a commercial publication might be limited, and an open access format would ensure that members of these laboratories could examine this material if it interested them. Although I do not have expertise in all the areas that I have covered, I used the opportunity to review the literature in the areas with which I was unfamiliar. I have attempted to interpret some features of baculovirus biology in terms of their hypothetical function. These theories are based on current and probably very limited understanding of the biology of this complex group of viruses, and I welcome future information that will explain these phenomena in more detail. Any comments or suggestions for revision of this text are also welcome, because I am certain that much has been overlooked, and with the availability of online publication, more complete future editions of this book are possible.

I am indebted to Mauro Martignoni and George Beaudreau for assisting me in starting research on baculoviruses back in the mid-1970's. They were always interested and enthusiastic, and without their encouragement and forbearance, it is unlikely that I would have had a career in baculovirology.

I would like to also express my appreciation to Drs. Margot Pearson and Victor Mikhailov for their reading and comments on this manuscript and also for the many contributions that they made to the understanding of baculovirus molecular biology over the years. The numerous individuals who answered my questions, shared their insight, and provided me with manuscripts before their publication are also greatly appreciated, as is the assistance of Desiree Rye of the NLM in editing the manuscript. This book could not have been produced without the encouragement, assistance, computer skills, and guidance of Dr. Laura Dean, also of the NLM.

Chapter 1

Introduction to the baculoviruses, their taxonomy, and evolution

The discovery of baculoviruses

The history of the discovery of baculoviruses is intimately related to the development of the silk industry that occurred in China as early as 5000 years ago. The culture of silkworms spread throughout Asia and reached Japan via Korea by about 300 C.E. and arrived in Europe by about 550 C.E. By the 12th century it was established in Italy and Spain and eventually spread to France and England and to Mexico by the 1500s. Silk production has been of major cultural significance in both China and Japan. It was a major item of commerce and in Japan is extensively documented in historic prints [1] and although no longer a major industry, silk production is still practiced symbolically by the Japanese royal family. (For a review of the history of silkworm culture, see [2]). As with any agricultural enterprise, problems were encountered caused by a variety of diseases, and these had to be confronted in order for the industry to flourish. Even before the germ theory of disease was introduced, a variety of different types of illnesses afflicting silkworms had been described, and methods to mitigate the effects of these diseases had been developed. With the advent of light microscopy, a prominent feature of one of the types of diseases was characterized by the presence of highly refractile occlusion bodies that were symptomatic of the affected insects. These were commonly polyhedron shaped (Fig. 1.1) and led to the naming of the diseases associated with these structures as 'polyhedroses' by the mid-1800s. Although the presence of infectious particles within occlusion bodies had been suggested earlier, it was not until the late 1940s that the presence of rod-shaped virions was convincingly demonstrated by electron microscopy [3]. This and other investigations also established the crystalline nature of the occlusion body structure. Subsequently, two different types of polyhedrosis diseases were distinguished; those in which the polyhedra developed in nuclei and were called nuclear polyhedroses (NPVs), and those with occlusion bodies present in the

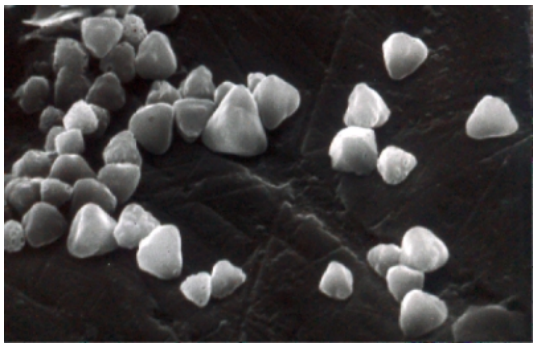

Fig. 1.1 Baculovirus occlusion bodies. Scanning EM by K. Hughes and R. B. Addison.

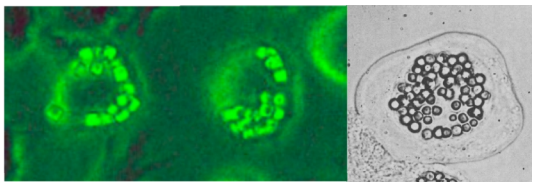

Fig. 1.2. Comparison of cells infected with a cypovirus (CPV – Reoviridae) and a baculovirus. Left panel: CPV of *Orgyia pseudotsugata*, Photo by G. S. Beaudreau. Right Panel: AcMNPV infected T. ni cells. From Volkman and Summers [65]. Copyright 1975 American Society for Microbiology. Reproduced with permission via Copyright Clearance Center.

cytoplasm (Fig. 1.2). These were called cytoplasmic polyhedroses (CPVs) [4]. In contrast to the rod-shaped, DNA-containing NPVs, the CPVs were observed to have icosahedral capsids and were placed in the Reoviridae (genus Cypovirus), a family of viruses with segmented double stranded RNA genomes. A second category of baculovirus characterized by the presence of small, granular, ellipsoidal-shaped occlusion bodies was originally reported in the 1920s [5] and was named granulosis viruses (GVs). The division of the baculoviruses into two major groups, the nuclear polyhedrosis viruses [now called nucleopolyhedroviruses (NPV)] (Fig. 1.3) and the granulosis viruses [now called granuloviruses (GVs)] (Fig. 1.4) based on occlusion body morphology defined the major

taxonomical divisions of these viruses until the advent of molecular biology.

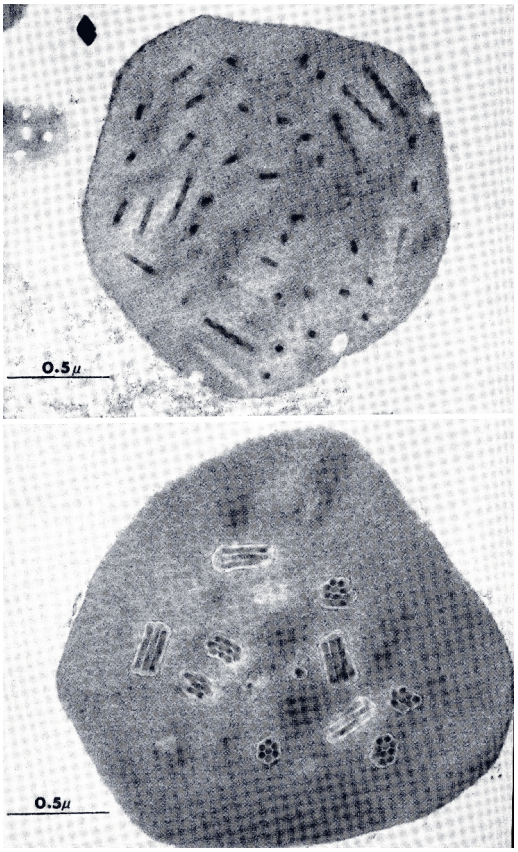

Fig. 1.3. Two nucleopolyhedroviruses pathogenic for *Orgyia pseudotsugata* showing single (top panel) and multiple (bottom panel) nucleocapsids/envelope. Top panel: OpSNPV. Bottom panel: OpMNPV. From Hughes and Addison [33]. Copyright 1970 Elsevier. Reproduced with permission via Copyright Clearance Center.

The terminology for these viruses went through a series of names, and it was not until 1973 that a nomenclature that included Borrelinavirus, Bergolidavirus, Smithiavirus, Moratorvirus and Vagoiavirus in honor of various historic individuals who had done early research on NPVs and GVs was changed and unified into the Baculoviridae [6]. The name baculovirus was proposed by Mauro Martignoni, who because of his Italian-Swiss heritage was a Latin scholar. He suggested that they be named baculoviruses (family = Baculoviridae) because of the rod-shape of their virions, which is derived from Latin *baculum* = cane, walking stick, staff.

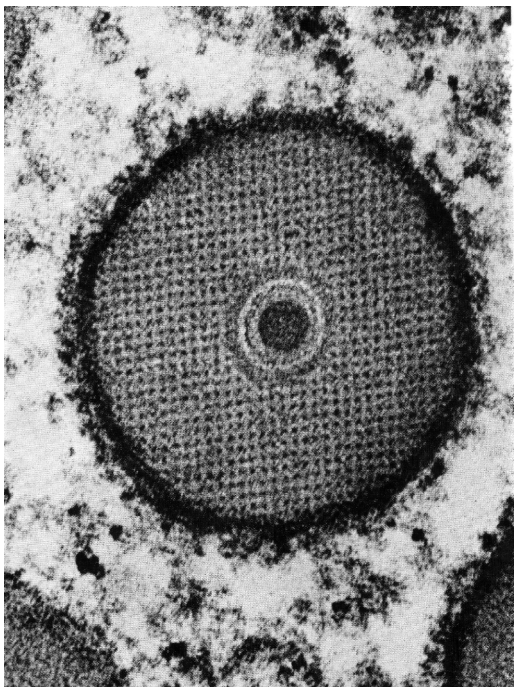

Fig. 1.4 Cross section of a granulovirus of *Plodia interpunctella*. From Arnott and Smith [66]. Copyright Elsevier 1967. Reproduced with permission via Copyright Clearance Center.

The significance of baculoviruses in Nature

Although much of the early interest in baculoviruses was due to the threat they posed to the silk industry, baculoviruses play a major role in the control of natural insect populations. For example, they are a major regulator of gypsy moth populations in North America and in some instances have been shown to be responsible for over 50% of the mortality observed [7]. They also are major contributors to the collapse of Douglas-fir tussock moth outbreaks [8]. In addition to forest insect populations, they also appear to be important in the natural control of agricultural pests of human food crops and as a result they may be a substantial contaminant of the human diet. For example, in one study it was found that cabbage purchased from 5 different supermarkets in the Washington D.C. area were all contaminated with baculoviruses to such an extent that each serving (about 100 cm^2 of leaf material) would contain up to 10^8 polyhedra of an NPV pathogenic for the cabbage looper, *Trichoplusia ni* [9]!

The definition is not in the name: Naming baculoviruses

Baculoviruses are normally named for the initial host from which they were isolated. Consequently, the type NPV species, AcMNPV, was named for its host, the alfalfa looper, *Autographa californica* (Ac). This naming would be straightforward except that AcMNPV infects a wide variety of lepidopteran insects and its name originated because of its initial association. Consequently, a variety of other virus isolates e.g., *Galleria mellonella* (GmNPV), *Rachiplusia ou* (RoNPV), and *Plutella xylostella* (PlxyNPV), although having unique names, are closely related (~96-98.5% at the amino acid sequence level) variants of AcMNPV. In addition, viruses were originally named by the first letter of the genus and species of their host. However, as more viruses were discovered, some infected different insects that had names with the same first letters. This resulted in different viruses with the same descriptor. Consequently, the first two letters of the genus and species have become the convention, i.e., AcMNPV should really be AucaMNPV. However, since AcMNPV, GmMNPV and RoMNPV have been used so extensively, the original abbreviations have been retained.

What defines a baculovirus?

Genomes and nucleocapsids

Baculoviruses are a very diverse group of viruses with double-stranded, circular, supercoiled genomes, with sizes varying from about 80 to over 180 kb and that encode between 90 and 180 genes. Of these genes, a common set of about 30 homologous genes has been identified, and there are probably others that cannot be recognized because of the extent of changes incorporated over time. The genome is packaged in rod-shaped nucleocapsids that are 230– 385 nm in length and 40 – 60 nm in diameter [10, 11]. In the most well characterized baculoviruses, the virions are present as two types, occluded virions (ODV) and budded virions (BV). Although these two types of virions are similar in their nucleocapsid structure, they differ in the origin and composition of their envelopes and their roles in the virus life cycle (see Chapter 2).

Occlusion bodies

Members of the Baculoviridae are characterized by their presence in occlusion bodies called polyhedra for NPVs and granules or capsules for GVs. Polyhedra are about 0.6 – 2 μM in diameter, whereas granules are oval-shaped with diameters of about 0.2-0.4 μM [10]. Occlusion bodies are highly stable and can resist most normal environmental conditions thereby allowing virions to remain infectious indefinitely. Evidence suggests that they can survive passage through the gastrointestinal tract of birds, which can facilitate their dispersal [12, 13]. The occlusion body consists of a crystalline matrix composed of a protein called polyhedrin in NPVs and granulin in GVs. Although they have different names, these two proteins are closely related.

Recently, nudiviruses, that may be occluded or non occluded have been shown to be related to baculoviruses [14]. Therefore in the future occlusion might not be considered a definitive feature of the Baculoviridae.

Baculovirus hosts

Over the years, baculoviruses have been reported from a variety of different species of invertebrates. However, the only well-documented hosts are Diptera, Hymenoptera, and Lepidoptera. Convincing documentation has been reported for occluded virions resembling NPVs in a caddis fly (Trichoptera) [15] and a shrimp species [16, 17]. An occluded baculovirus-like virus was also reported for a thysanuran, but it did not appear to affect its host and transmission studies failed [18]. Baculoviruses have also been reported from Orthoptera [19], but later these were classified as pox viruses, and from Coleoptera, but these are normally not occluded and were later placed in an unassigned category. Reports of infection of other insects, e.g., a coleopteran [20] could not be confirmed (Rohrmann, unpublished). In addition, there is a report of a baculovirus infecting a neuropteran [21]. However, the infection occurred under laboratory conditions, where neuropterans were fed on lepidoptera that had died of an NPV infection. Consequently, the neuropterans were likely heavily contaminated from their food source, and although they appeared to die of an NPV infection, they were probably exposed to an unusually high virus dose. Naturally infected Neuroptera have not been documented. An example of the

distribution of baculoviruses in insects that has been reported in the literature [22] is shown in Table 1.1. These numbers should be viewed with caution because many of the reports could be of the same virus infecting different species. However, they do give a good general overview of the likely distribution of baculoviruses. Of particular note is that GVs are confined to the Lepidoptera. In addition, all the hymenopteran hosts belong to a suborder called Symphyta that are comprised of sawflies (named because their ovipositor resembles a saw and in some species is used to cut into plants for egg deposition). Sawflies resemble Lepidoptera; they have herbivorous, caterpillar-like larvae and are distinct from the other hymenopteran suborder, Apocrita, which includes bees, ants, and wasps.

Table 1.1. Distribution[1] of baculoviruses in insect orders [22].

Insect orders	NPVs	GVs
Diptera	27	
Hymenoptera (Symphyta)	30	
Lepidoptera	456	148

[1]Indicates the number of species that have been reported to be infected

Other defining features of the Baculoviridae: a virus encoded RNA polymerase

In addition to invertebrate hosts, circular, supercoiled double-stranded DNA genomes, rod-shaped enveloped nucleocapsids, and the production of occluded virions, an additional defining feature of baculoviruses is that they encode their own RNA polymerase. The core enzyme is composed of four subunits and functions in the transcription of late promoters that are initiated within a novel sequence element (see Chapter 6). Whereas other DNA viruses of eukaryotes encode their own polymerase, e.g., poxviruses, they replicate in the cytoplasm and therefore do not have access to the host cell transcriptional apparatus located in nuclei. Baculoviruses, in contrast, exploit the host cell transcriptional system for expressing their early genes, but after initiation of DNA replication they are dependent upon their own RNA polymerase for transcription of their late and very late genes.

Baculovirus Diversity

The recent proliferation of baculovirus genome sequences has greatly expanded our understanding of their diversity and evolution. This has yielded distinct patterns of virus relatedness (Fig. 1.5) in which virus lineages are

Table 1.2 Genera of the Baculoviridae

Genus	Members
Alphabaculovirus	Lepidopteran NPVs
Betabaculovirus	Lepidopteran GVs
Gammabaculovirus	Hymenopteran NPVs
Deltabaculovirus	Dipteran NPVs

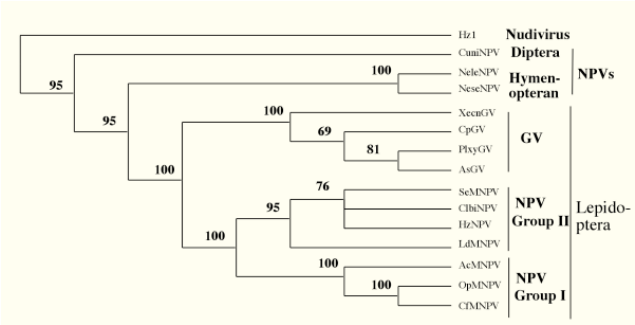

Fig. 1.5. Phylogenetic relatedness of LEF8 from selected baculoviruses. Neighbor joining; bootstrap analysis (1000 reps).

associated by the host that they infect. The Baculoviridae are divided into four genera [23]. The viruses of Lepidoptera are divided into Alpha- and Betabaculoviruses encompassing the NPVs and GVs, respectively, and those infecting Hymenoptera and Diptera would be named Gamma- and Deltabaculoviruses, respectively (Table 1.2). Such patterns of host-associated virus diversity were first observed for small DNA viruses (papilloma and polyoma viruses) of mammals [24] and suggested for baculoviruses based on N-terminal polyhedrin and granulin sequences [25]. In this process, viruses associate with a host and as their host becomes genetically isolated and speciates, so does the virus in a process called host-dependent evolution. As more sequence data has become available, this process has been more convincingly demonstrated for baculoviruses [26] and is clearly reflected in Fig. 1.5 in which the major lineages are clustered into clades based on the host insect that they infect. In GVs, host-dependent evolution has been suggested on the level of insect families [27]. A major division has been observed in the lepidopteran NPVs that has resulted in the separation of this lineage into two major Groups, I and II [28]. These two groups differ significantly in gene content, most notably Group I NPVs use GP64 as their budded virus (BV) fusion protein, whereas Group II NPVs lack *gp64* and utilize a protein called F [29]. There

are also 11 other genes in addition to *gp64* that appear to be found only in Group I NPVs (Table 1.3). It has been suggested that the Group I lineage originated when a NPV variant uniquely containing these genes incorporated *gp64* which stimulated their evolution as a distinct lineage [30] [31]. Although the list of host insects provides information on baculovirus distribution, sequence analysis of common sets of genes from a wide variety of baculoviruses has provided a picture of the actual extent of their diversity [32]. From this data, a picture of the lepidopteran viruses has emerged. However, more information on viruses from the other insect orders is needed to complete our understanding of baculovirus evolution.

Table 1.3. Genes[1] found in and unique to all sequenced Group I NPV genomes

Ac1 (ptp), Ac5, Ac16 (BV-ODV26), Ac27 (iap-1), Ac30, Ac72, Ac73, Ac114, Ac124, Ac128 (gp64), Ac 132, Ac151 (ie2)
[1]Genes are designated by their AcMNPV orf number

Multiple versus single nucleocapsids

A prominent feature of the nucleocapsids within polyhedra is their organization into either single or multiple aggregates of nucleocapsids within an envelope (Fig. 1.3). For example, in some NPVs there can be from 1 to 15 nucleocapsids per envelope, with bundles of 5 to 15 predominating. In contrast, strains defined as having a single nucleocapsid per envelope rarely show more than one nucleocapsid per envelope [10, 33]. Because this feature is so distinctive and characteristic of specific isolates, it was incorporated into the early nomenclature such that NPVs were categorized as either MNPV or SNPVs (also previously called multiply or singly embedded virions [MEV and SEV]). In addition, whereas MNPVs and SNPVs were both found in lepidopteran viruses, only SNPVs were observed in other insect orders. GVs were also categorized as singly enveloped; however, multiple GVs, although rare, have been described [34]. With the accumulation of DNA sequence data that allowed for the determination of definitive phylogenetic relationships, it was found that the MNPV and SNPV division did not conform to the phylogeny of the viruses. For example, at one point BmNPV was considered the type virus for SNPVs because of its production of predominantly single nucleocapsids [35]. However, sequence data indicates that BmNPV is closely related to AcMNPV (both belong to Group I), whereas other MNPVs such as LdMNPV and SeMNPV are more distantly related Group II viruses, which also includes SNPV-type viruses. Drawing conclusions regarding the properties of MNPV and SNPV viruses is fraught with difficulty because their genetic content is often so different that it is not possible to attribute characteristics of a virus to its M- or SNPV phenotype.

It was recently observed that AcMNPV deleted for Ac23, a homolog of the F fusion proteins of Group II viruses, show an elevated percentage of singly enveloped virions (45% vs 11- 22%) for different virus constructs encoding Ac23 [36]. Although it is a BV envelope protein, Ac23 has been reported to also be associated with occluded virions [37], although the ortholog from OpMNPV (Op21) did not appear to be ODV-associated [38]. It was suggested that these different observations were due to the use of a proteinase inhibitor for the characterization of Ac23 [37]. It has also been shown that deletion of Ac92, a gene encoding a sulfhydryl oxidase, results in virions that superficially resemble the SNPV phenotype [39]. However, they are not infectious indicating that they are very different from true SNPVs.

Despite agreement that the MNPV and SNPV designation is not a useful taxonomical trait, it continues to be employed, in part for historical continuity, and also because it can be a convenient method for distinguishing different viruses that are pathogenic for the same host, e.g., OpMNPV and OpSNPV which both infect *Orgyia pseudotsugata* (Fig. 1.3), but are members of Group I and II, respectively.

Gene content and organization

Although there are major differences in the sizes of baculovirus genomes, a few patterns are evident (Table 1.4). The viruses of members of the Hymenoptera contain the smallest genomes at a little over 80 kb. In contrast, the GV genomes vary from 101 kb (PlxyGV) up to 178 kb (XecnGV). Group I NPVs cluster around 130 kb, whereas Group II show a much higher degree of diversity, varying from about 130 to almost 170 kb. The small size of the hymenopteran NPV genomes might be attributed to a restricted life

cycle confined to replication in insect gut cells [40]. Consequently, unlike other baculoviruses that cause systemic infections, genes required for spread throughout the insect, where the virus encounters and replicates in a variety of tissues, might not be necessary. In contrast, the large size of a Group II NPV (LdMNPV), which at 161 kb is about 30 kb larger than many other NPV genomes, can be attributed to a combination of repetitive genes (16 *bro* [baculovirus repeated orf] genes) that add about half of the additional 30 kb, while genes not found in smaller genomes add most of the remainder. These additional genes encode ribonucleotide reductase subunits and two enhancin genes [41]. In other viruses with large genomes (e.g., XecnGV), repeated genes including 10 bro and 4 enhancin genes, comprise up to 17% (30 kb) of the genome [42]. Despite the large difference in gene content in GV genomes as reflected in their size range, their genomes are surprisingly collinear [43]. In contrast, NPVs even from the same order (Lepidoptera) show a high degree of variation [44].

Regulatory content: homologous regions (*Hrs*)

In addition to containing a set of genes encoding proteins required for productive infection, most baculovirus genomes also contain homologous repeated regions. In AcMNPV, *hrs* are comprised of repeated units of about 70-bp with an imperfect 30-bp palindrome near their center. *Hrs* are repeated at eight locations in the genome with 2 to 8 repeats at each site (see Chapter 4). They are highly variable and although they are closely related within a genome, they may show very limited homology between different viruses. For example, in the CpGV genome, tandem repeated sequences are not evident, although a 75-bp imperfect palindrome is present at 13 different locations on the genome [45]. In addition, in the TnSNPV (group II) sequence, *hrs* were not found [46]. *Hrs* have been implicated as both transcriptional enhancers and origins of DNA replication for some baculoviruses [47-52]

Conservation of baculovirus genes: core genes

Despite the diversity in gene content present in different baculovirus genomes, a set of about 31 genes that are present in all sequenced baculovirus genomes has been identified (Table 1.5). This set should be viewed with caution, however, as it is likely that many shared genes have diverged to such an extent that their relatedness cannot be readily determined. This is particularly true of the dipteran virus that is most distant from the baculoviruses of Lepidoptera and Hymenoptera. Of the conserved genes, about half are virion-associated proteins that are involved in capsid structure, the occlusion-derived virus envelope, and larval infectivity. Most of the others are related to DNA replication or processing, and late or very late transcription. Despite this limited set of genes that baculoviruses share, they do provide insight into some of the major functions required of all baculoviruses such as a common virion structure, the necessity to infect gut cells, and the use of a novel polymerase for the expression of late

Table 1.4. Genome size and predicted ORF content* of selected baculoviruses

Virus type	Name of Virus	Size (kb)	Orfs (>50 aa)
Group I (13 members)**	EppoMNPV [67]	119	136
	AnpeNPV [68]	126	145
	AcMNPV [69]	134	~150
Group II (20 members)	AdhoNPV [70]	113	125
	SeMNPV[71]	136	139
	AgseNPV [72]	148	153
	LdMNPV[41]	161	163
	LeseNPV [73]	168	169
GV (12 members)	AdorGV [74]	100	119
	CrleGV [43]	111	124
	CpGV [45]	124	143
	XecnGV [42]	179	181
Hymenopt. NPV (3 members)	NeleNPV [75]	82	89
	NeabNPV [76]	84	93
	NeseNPV [77]	86	90
Dipteran NPV (1 member)	CuniNPV [78]	108	109

*Selected from over 50 genome sequences (2010)

**The numbers in brackets indicate the total number of genomes in the category.

genes. Furthermore, there are sets of genes specific to, and conserved in, each clade of viruses such as gp64 in Group I NPVs and a number of GV-specific genes (see below). These genes likely reflect major recombination events that altered the properties of the viruses to such an extent that they subsequently developed along distinctive phylogenetic pathways. The observation that different types of NPVs and GVs and other types of DNA viruses (e.g., entomopox, ascovirus, nudivirus) have the potential to simultaneously infect the same insect indicates how via recombination, genes can be transferred between different viruses and between viruses and the host insect.

Lepidopteran baculovirus (NPV and GV) core genes

In addition to the core genes found in all baculoviruses, lepidopteran baculoviruses encode an additional set of genes that appear to be present in all their genomes and about half are also found in Gammabaculovirus (hymenopteran virus) Table 1.6 Additional Core genes present in all Lepidopteran (Alpha- and Beta-) and some Gammabaculoviruses genomes (Table 1.6). Homologs, or functional analogs of many of these genes are likely present in all baculoviruses, but the relationships can not be detected because of the extent of divergence the genes. Since they appear to be so central to the replication of the baculoviruses, the single stranded DNA binding protein, LEF-3, and the transcriptional activator IE-1 may be examples of gene divergence which prevents their detection in the more distantly related viruses. In contrast, homologs of Ac38, an ADP-ribose phosphatase, belong to a family found in many organisms and would likely be detected outside the lepidopteran baculoviruses, if it were present. It acts as a decapping enzyme in vaccinia virus [53] and its presence suggests that the lepidopteran viruses employ molecular strategies significantly different from the other baculovirus genera. Polyhedrin appears to be an example of possible convergent evolution since the occlusion body protein of the dipteran virus appears to encode a novel protein [54]. A few genes are present in all Alphabaculoviruses but are found in some, but not all, Betabaculoviruses (Table 1.7). The most well-characterized of these Ac9 (orf1629) which is a homolog of WASP proteins that are involved in movement of the virus based on actin polymerization (see Chapter 3). This would suggest that other baculoviruses have major differences in their pathogenesis.

Table 1.5. Conserved genes[1] in baculoviruses and nudiviruses

Conserved in baculovirus	Conserved in Nudivirus
AcMNPV ORF	
Ac6 Lef2	
Ac14 Lef1	
Ac22 PIF2	+
Ac40 p47	
Ac50 LEF-8	+
Ac54 vp1054	
Ac62 LEF9	+
Ac65 DNA pol	+
Ac66	+(GbNV)
Ac68	
Ac77 VLF-1	+
Ac80 gp41	+
Ac81	+
Ac83 VP91	+
Ac89 VP39	+
Ac90 LEF-4	+
Ac92 p33	+
Ac95 DNA helicase	+
Ac96 (PIF-4)	+
Ac98	+
Ac99 LEF-5	+
Ac100 p6.9	
Ac109	
Ac115 PIF-3	+
Ac119 PIF-1	+
Ac133 Alkaline nuclease	
Ac138 p74	+
Ac142	
Ac143 odv-E18	
Ac144	
Ac148 odv-e56 (PIF-5)	+

[1]For more details see Chapter 12

Granulovirus-specific genes

In addition to their occlusion body morphology, the pathology of GV infection differs from NPVs. For example, their replication is not confined to nuclei as the nucleus and cytoplasm appear to merge during GV infections. The presence of GV specific genes may be a reflection of these differences. Granuloviruses encode a number of genes that are found in all GV genomes. Of these GV genes, 14 appear to be specific to GVs,

whereas others, although found in all GV genomes, are also found in a few other baculoviruses. Examples of the latter are DNA ligase and helicase-2 homologs that, in addition to GV genomes, are also present in one NPV genome (LdMNPV). The helicase-2 homolog is also found in an additional NPV (Maco-B-NPV). There are other GV-specific genes that are found in most, but not all, GV genomes. The only GV specific gene that has been characterized is the XecnGV metalloproteinase. It was found to have proteinase activity and was inhibited by metalloproteinase inhibitors [55].

Table 1.6 Additional Core genes present in all Lepidopteran (Alpha- and Beta-) and some Gammabaculoviruses

AcMNPV orf	Name /function	Presence in Gamma-
Ac8	polyhedrin	+
Ac10	pk-1	
Ac13		
Ac23	related to fusion (F) protein	
Ac25	DNA binding protein, dbp	+
Ac28	lef-6	
Ac29		
Ac32	fgf	
Ac36	Pp31/39K	
Ac37	LEF-11	+
Ac46	Odv-E66	
Ac38	ADP-ribose phosphatase, nudix	
Ac53a	LEF-10	
Ac61	Few polyhedra, fp	
Ac67	LEF-3	
Ac75		+
Ac76		+
Ac78		+ (in 2 of 3)
Ac82	Telokin-like	
Ac93		+
Ac94	ODV-E25	
Ac101	BV/ODV-C42	+
Ac102		
Ac103		+
Ac106/107		+
Ac110		
Ac139	ME53	
Ac145		+
Ac146	Ie-1	
Ac147	Ie-0	

Table 1.7 Genes present in all Alphabaculoviruses (lepidopteran NPVs) and some Betabaculoviruses (GVs)

AcMNPV orf	Name /function	Presence in Beta
Ac9	Pp78/83; orf1629	
Ac15	egt	Most Beta
Ac24	Protein kinase interacting protein (PKIP)	
Ac34		
Ac60	Chab-like	Most Beta
Ac104	Vp80capsid,vp87	
Ac131	Calyx, polyhedron envelope	
Ac141	Exon-0	Some Beta

Related viruses: the nudiviruses

In addition to baculoviruses, there are a number of viruses pathogenic for invertebrates that have large double-stranded DNA genomes. These include nudiviruses, ascoviruses, irridoviruses, and entomopox viruses. However, with the exception of nudiviruses, all these viruses replicate in the cytoplasm or exhibit a combination of both nuclear and cytoplasmic replication (ascoviruses) (reviewed in [56]). In addition, although nudiviruses are an unassigned genus of viruses, they closely resemble baculoviruses and were originally classified as non-occluded baculoviruses, although occlusion bodies have been occasionally reported (e.g., see [57]). Their similarity to baculoviruses includes nuclear replication and rod-shaped, enveloped, nucleocapsids. They have double strand, circular DNA genomes varying in size from 97 - 230 kb [14]. They were removed from the Baculoviridae because they are nonoccluded. Hence the etymology of their name (latin *nudi* = naked, bare, uncovered), because they lack occlusion bodies. They also have a somewhat different host range than baculoviruses having been characterized not only from Lepidoptera, but also from Coleoptera (a rhinoceros beetle), and Orthoptera (a cricket).

As more genome sequence data has become available, homologs of baculovirus genes have been found in many different categories of invertebrate viruses. These appear to be the

result of lateral transfer, probably during co-infection of the same host [58]. However, the nudiviruses share a significant number of the conserved core genes found in all baculoviruses and therefore their presence does not appear to be a result of lateral gene transfer. As shown in Table 1.5, nudivirus genomes contain homologs of 20 of the 31 core conserved baculovirus genes. These include homologs of genes encoding the novel baculovirus RNA polymerase subunits, virion structural proteins, and a set of genes required for infection of gut tissue. In addition, genes such as DNA helicase are present in many large DNA virus genomes, and LEF-8 has homology to RNA polymerase subunits from many organisms. However, homology searches group these genes from baculoviruses and nudiviruses together. Therefore, based on morphology and molecular phylogeny, these two groups of viruses clearly share membership in a distinct viral lineage.

An occluded virus of pink shrimp, *Panaeus monodon*

An occluded baculovirus-like element pathogenic for the pink shrimp *P. monodon* has been described [16, 17]. These viruses produce striking pyramidal-shaped occlusion bodies containing rod-shaped nucleocapsids similar in dimension and appearance to those of baculoviruses (Fig. 1.6). Analysis of several sequences from this virus has indicated that it encodes proteins similar to the baculovirus/nudivirus lineage including LEF-5, LEF-9 and VLF-1 [14, 59] (Fig. 1.7). Completion of the sequence of this genome should definitively clarify its phylogeny.

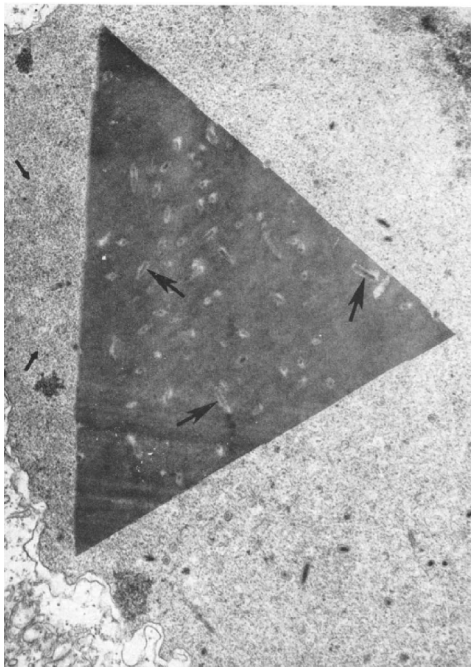

Fig. 1.6. Polyhedral inclusion body of a virus of the pink shrimp, *Penaeus monodon*. Arrows indicated nucleocapsids. From Couch [16]. Reproduced with permission of Elsevier Limited via Copyright Clearance Center.

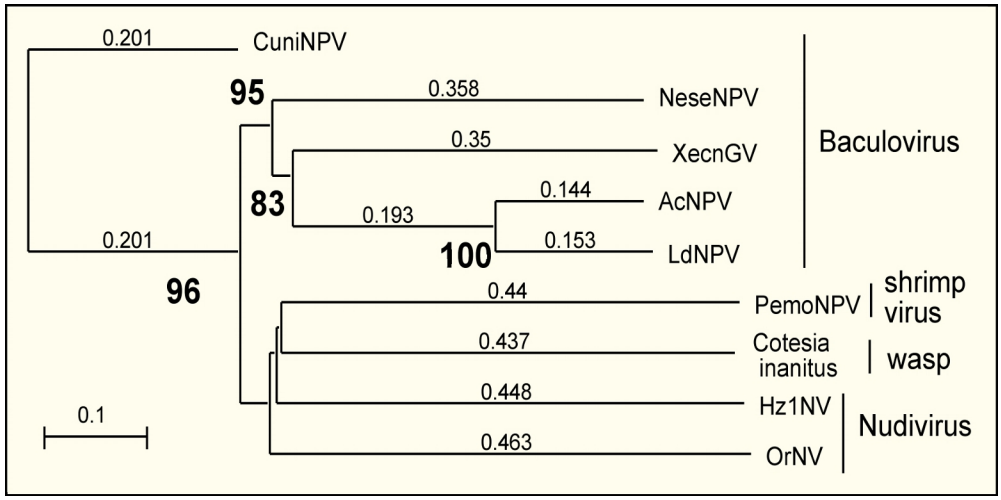

Fig. 1.7. Phylogenetic relatedness of VLF-1 from selected viruses and a braconid wasp. Neighbor joining Best Tree; Numbers in bold indicate Bootstrap values (1000 reps) over 50%. Abbreviations: PemoNPV, Penaeus monodon (shrimp); *Cotesia inanitus* (braconid wasp); Hz1NV and OrNV, nudiviruses.

A polydnavirus connection

Polydnaviruses are not true viruses because they do not contain genetic material for their own replication. They are produced in the ovaries of parasitic wasps and the virion-like particles contain genetic material from the wasp and are injected into host lepidopteran larvae along with the wasp eggs. They are named because they contain multiple circular double-stranded DNA molecules (polydispersed DNA) encompassing up 560kb that encode gene products that compromise the target host immune system and other processes and are essential for the successful development of the wasp egg. There are two lineages of parasitic wasps that employ these elements; the braconid and ichneumonids and they produce bracovirus and ichnovirus polydnavirus elements, respectively. When cDNAs produced from RNA associated with polydnavirus production in braconid ovaries were analyzed, a number were found to exhibit sequence homology to genes encoded by nudiviruses and baculoviruses (Fig. 1.7). These included per os infectivity factors that are involved in infection of mid-gut cells by baculoviruses. Another group of factors are related to subunits of the baculovirus RNA polymerase and a very late transcription factor, VLF-1. When the structural proteins of the polydnavirus virions was analyzed, proteins related to per os infectivity factors were also identified. The presence of homologs of these factors suggests that they may be involved in a similar role to that played in baculoviruses by delivering the bracovirus DNA into cells of the parasitized larvae. The logic of the production of host the viral RNA polymerase subunits in association with virus production remains to be explained [60, 61]. These data have been interpreted to indicate that a parasitic wasp was infected by a nudivirus and all or part of the nudivirus genome was incorporated into the wasp genome. Some of these genes were subsequently adapted for the packaging of the polydnavirus DNA thereby facilitating their transmission into the host larvae. A similar analysis of an ichnovirus found that polydnavirion-associated proteins did not show relatedness to known proteins. However, because the genes encoding these products were found clustered in specific locations and are amplified along with the DNA that is packaged in the ichnovirus virions, it was suggested that they are derived from a hitherto unknown virus that integrated into the host genome similar to the nudivirus-like element that is present in the genomes of braconid wasps [62].

A phylogenetic tree of VLF-1 from representative baculovirus, nudivirus, polydnavirus (bracovirus) host (*Cotesia inanitus*), and two nudiviruses is shown in Fig. 1.7. Although there appear to be two distinct lineages with the baculovirus comprising one and the other including the nudiviruses, shrimp virus, and bracovirus host (*Cotesia*), the latter branch is not well supported and therefore it is difficult to draw conclusions regarding the relationships of the non-baculovirus lineages.

Hytrosaviruses and a Whispovirus

Hytrosaviruses include the salivary gland hypertrophy viruses and have been characterized from several Diptera including the tsetse fly, the vector for sleeping sickness. They a non-occluded and contain large double stranded DNA genomes and have a virion morphology similar in both size and appearance to baculoviruses. They appear to infect the salivary gland and although not particularly virulent, they can result in a significant reduction in reproductive fitness [63].

The genus Whispovirus includes the white spot syndrome virus (WSSV) that causes severe disease outbreaks in cultured penaeid shrimp, particularly in Asia. It is a non-occluded, enveloped, rod-shaped virus with a double stranded DNA genome of up to 300 kb. It is a highly virulent virus and causes major tissue damage and the infection results in white spots of calcium deposited in the shell [64].

Genomes of both the WSSV and several hytrosaviruses have been sequenced and have been found to encode homologs of four baculovirus/nudivirus genes involved in per os infectivity including pifs 1-3 and p74 [14]. It is unclear if these viruses have a direct phylogenetic relatedness to the baculovirus/nudivirus lineage, or whether this is an example of a set of genes important in oral infectivity that was transferred between viruses. The lack of RNA polymerase subunits suggests that their molecular biology may be significantly different from the baculovirus/nudivirus lineage.

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Chapter 2

Structural proteins of baculovirus occlusion bodies and virions

It has long been recognized that infectious baculoviruses can be generated from the transfection of naked viral DNA into susceptible insects cells and that the transcription of early genes required for initiating the infection is accomplished via the host cell RNA polymerase. Therefore, viral structural proteins, although necessary for protecting the viral genome and initiating infection, very likely are not required for functions such as transcription or DNA replication. Below is an overview of the proteins that have been associated with major baculovirus virion structures. These include occlusion bodies, budded virus (BV) and occlusion derived virus (ODV) envelopes, and proteins that are components of nucleocapsids. Most of the latter categories are likely shared between BV and ODV.

Occlusion body evolution

In temperate climates and in many tropical areas where there are wet and dry seasons, insect populations are transitory and expand dramatically during warm, moist periods, and then collapse with the onset of colder temperatures or drought when food sources are reduced or eliminated. In addition, even during seasons optimal for insect growth and reproduction, their populations are normally limited by predators, pathogens, normal temperature cycles, and food sources. However, under certain circumstances, insect populations can expand dramatically when a combination of conditions greatly increases their food supply, facilitates high levels of reproduction, or eliminates predators and pathogens. These cycles of population expansion are well documented for forest insects and may be separated by long periods of time. For example, the Douglas fir tussock moth, a pest of forests in Western North America has cycles of 7 to 10 years, and other insect epizootics are separated by 5 to 40 years (reviewed in Ref. [1]). Viruses often play a major role in causing the collapse of these large insect populations.

A major consequence of the cyclic nature of insect populations is that their pathogens are left without hosts either seasonally, or for much longer periods of time. Viruses, in general, have developed several methods to insure their survival until their hosts reappear. If their hosts are seasonal, such as those of insect pathogens, viruses could be present in the eggs or pupae of over wintering insects, or they might persist in alternate hosts, or some might remain stable outside their host. Although there is some evidence for baculoviral persistence within host insects (see Chapter 3), stability outside their host mediated by their presence within occlusion bodies appears to be a common feature of all baculoviruses. By immobilizing the virus within the crystalline protein lattice of the occlusion body, an environment is provided that allows virions to remain viable indefinitely as long as they are protected from extremes of heat and from UV light. In addition, the stability provided by occlusion may be of such a selective advantage for insect viruses that it has apparently been incorporated into the life cycle of three different types of insect viruses; in Reoviridae (cypoviruses, double-stranded RNA viruses with segmented genomes), the Poxviridae (entomopox viruses-dsDNA viruses with cytoplasmic replication), and in the Baculoviridae. No primary amino acid sequence relatedness is evident between these different categories of occlusion body proteins. Furthermore, the occlusion body protein of a dipteran baculovirus appears to be unrelated to other baculovirus polyhedrins [2] and the proteins from some cypoviruses also appear to be unrelated to each other. Similarly, the occlusion body protein of a baculovirus-like pathogen of the pink shrimp, *Penaeus monodon* did not appear to be related to baculovirus polyhedrin [3], even though other sequence data suggested the virus was related to the baculovirus lineage [4]. The

structure of both cypovirus and baculovirus polyhedrins have been determined and they appear to be unrelated and therefore are examples of convergent evolution [5, 6]

Although polyhedra are thought to stabilize baculovirus virions, in one example it was estimated that only about 0.16% of OpMNPV occlusion bodies remained infectious one year after an epizootic. However, considering that over 3 x 10^{15} occlusion bodies were estimated to have been produced per hectare during the outbreak, this would still be a substantial number of infectious viruses (reviewed in Ref. [1]).

Table 2.1 Proteins Associated with Baculovirus Occlusion Bodies

AcMNPV orf # and name	Distribution in the Baculoviridae	Effect of Deletion[2]
Ac8 Polyhedrin	All[1]	Viable
Ac131 Polyhedron envelope/Calyx	All except CuniNPV	Viable
Enhancin	A few NPVs and GVs	Viable
Ac137 p10	Group I/II; some GVs	Viable
Alkaline proteases	Non baculovirus contaminants	
[1]CuniNPV polyhedrin is unrelated to that of other baculoviruses. [2] For details see Chapter 12		

Occlusion body proteins

In addition to virions, occlusion bodies are composed of the matrix that occludes the virions and an outer membrane-like structure on the surface. Several other proteins are also associated with polyhedra (Table 2.1). The major protein forming occlusion bodies is polyhedrin/granulin.

Polyhedrin/Granulin. Polyhedrin and granulin are closely related and are the major structural components of occlusion bodies (polyhedrin in NPVs and granulin in GVs). They initially were challenging to characterize because the occlusion bodies are naturally contaminated with a protease (see below). However, once it was found that the proteases could be heat inactivated, polyhedrins from several viruses were characterized and were found to contain about 250 aa (30 kDa) and are one of the most conserved baculovirus proteins. They form a crystalline cubic lattice that is interrupted by and surrounds embedded virions (Fig. 2.1). Orthologs of polyhedrin/granulin are found in all baculovirus genomes, except for that of the dipteran virus (CuniNPV). Surprisingly, CuniNPV has an occlusion body protein that appears to be unrelated in primary amino acid sequence to the polyhedrin of other baculoviruses and is about three times as large [2, 7]. The polyhedrin of an NPV pathogenic for pink shrimp also appears to be unrelated to that from other baculoviruses in its primary sequence [3]. However, it was reported that it did react with antiserum to AcMNPV and TnGV polyhedrin even though at 50 kDa it appears to be twice as large as the insect baculovirus polyhedrins [8].

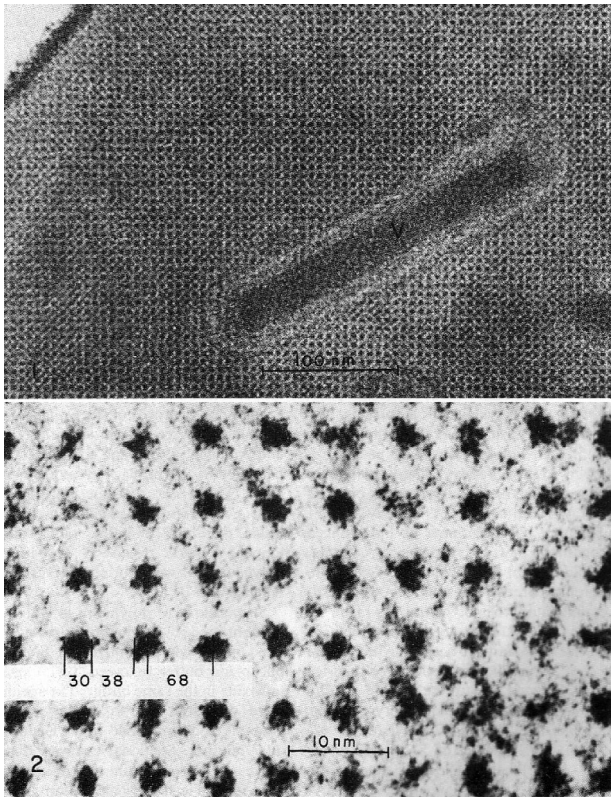

Fig. 2.1. Sections of portions of polyhedra showing crystalline structure. Top panel is from an NPV of *Pseudohazis eglanterina*, (pine butterfly). Bottom panel is a higher magnification from an NPV of *Nepytia freemani* (false hemlock looper). Measurements are shown in Angstroms. From K. Hughes [168]. Reproduced with permission of Elsevier Limited via Copyright Clearance Center.

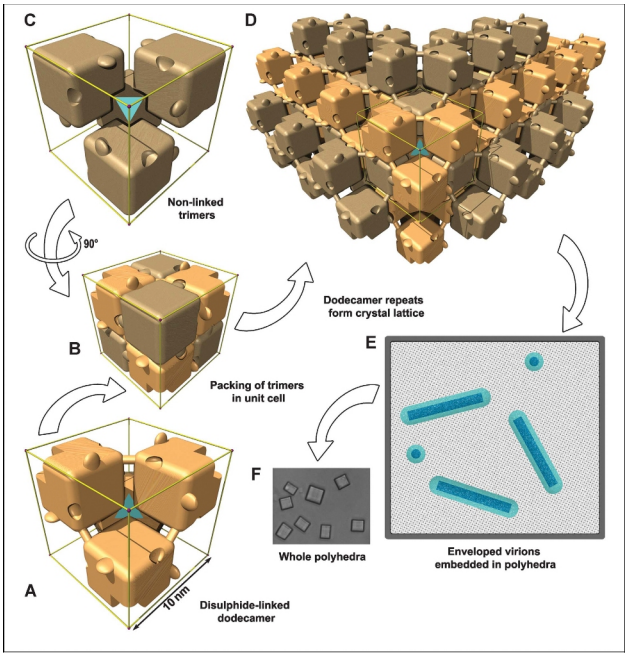

Fig. 2.2. The assembly of polyhedrin into polyhedra. Polyhedrin trimers are depicted as simplified cubic blocks. To clarify interpretation, the edges of the unit cell are shown in gold and a cyan tetrahedron symbolizes the cell centre. Within a unit cell, disulphide-linked trimers with one polarity are coloured light beech (A) and those with the opposite polarity are coloured light brown (C). (B) All eight trimers in the unit cell. The disulphide bond connecting adjoining trimers is shown as a dowel. (D) The crystal lattice is built up from repeats of the dodecameric unit. (E) Sketch of a cross-section through a polyhedron. The lattice spacing of the unit cells is illustrated as a dot pattern into which are embedded nucleocapsids (dark blue) surrounded by an envelope (cyan). (F) Light microscopy image of G25D mutant AcMNPV polyhedra. From Ji et al [10]. Copyright 2008 by Nature Publishing Group. Reproduced with permission of Nature Publishing Group via Copyright Clearance Center".

Despite forming a natural crystal in nature, determining the structure of polyhedrin by crystallography was unsuccessful because it was not possible to recrystallize it from solution. However, advances in crystallography, plus the observation that a single amino acid change (G25D in AcMNPV polyhedrin) resulted in larger than normal crystals [9] recently allowed the determination of the structure of polyhedrins from two different Alphabaculoviruses using occlusion bodies produced by viral infection [6] [10]. The 30 kDa subunits form trimers that are then arranged into dodecamers (four trimers) via disulfide bonds. This structure interlocks with another dodecamer to form the cubic-shaped unit cell of the crystal (Fig. 2.2). Hydrophobic and salt bridge interactions between the cubes likely form the linkages at the crystal interfaces that are disrupted by the alkaline pH of the insect midgut (see below). Occlusion of virion bundles might displace up to 20,000 polyhedrin subunits and could destabilize the crystals. This possibly led to the evolution of the envelope structure that is thought to stabilize these structures [10]. The presence of disulfide bonds may have prevented the attempts to crystallize purified polyhedrin.

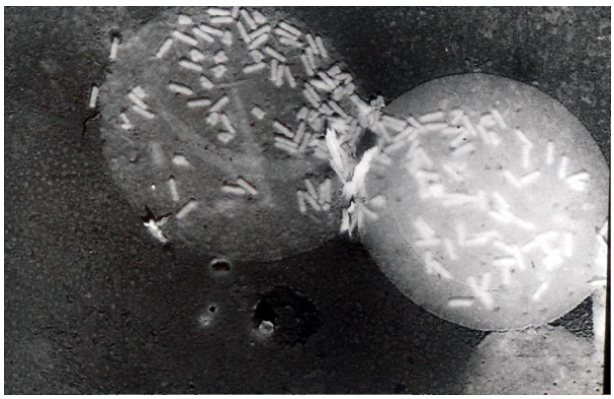

Fig. 2.3. Two adjacent dissolved polyhedra showing rod-shaped virions trapped by the collapsed polyhedron envelope. Photo by K. Hughes.

The calyx/polyhedron envelope (PE). The calyx/polyhedron envelope is an electron dense structure that forms a smooth, seamless surface that surrounds polyhedra. The function of the calyx/PE appears to be to seal the surface of polyhedra and to enhance their stability. In the laboratory when polyhedra are subjected to alkaline treatment, the crystalline lattice is dissolved, but the polyhedron envelope remains as a bag-like structure in which many virions are trapped (Fig. 2.3). Although the calyx/polyhedron envelope was originally found to be composed of carbohydrate [11], a phosphorylated protein component was subsequently identified [12]. This protein appears to be an integral component of the calyx/PE [13]. Homologs of the PE protein (Ac131) are found in the genomes of all lepidopteran NPVs. It is likely that when polyhedra are ingested by susceptible insects, they are dismantled by a combination of the

alkaline pH of the insect midgut and proteinases that are present in the midgut and associated with polyhedra. This combination would likely contribute to the disruption of the polyhedron and polyhedron envelope to facilitate virion release. The PE protein is associated with p10 fibrillar structures (Fig. 2.4) and p10 appears to be required for the proper assembly of the polyhedron envelope [14-17]. Polyhedra from viruses with either the p10 or PE gene deleted have a similar appearance; they have a rough pitted surface and the PE appears to be fragmented or absent (Fig. 2.5). In addition, in some viral genomes, genes are present that appear to be fusions of both PE and p10 protein domains further suggesting a fundamental relationship between these two proteins (see below).

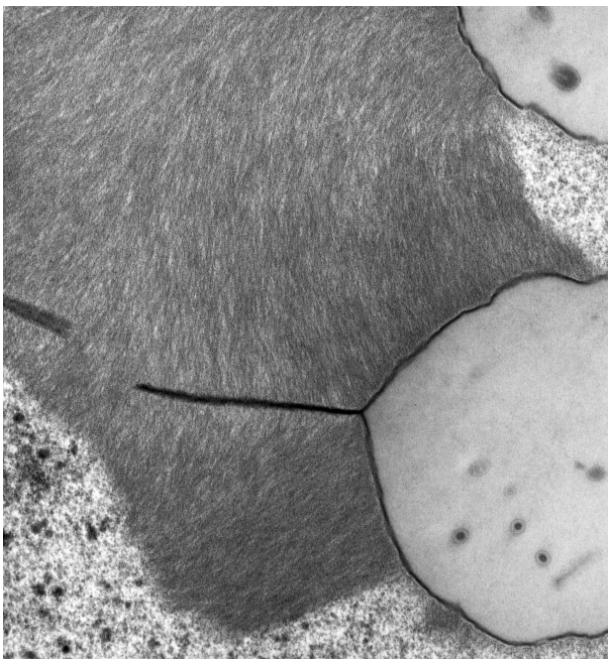

Fig. 2.4. Fibrous p10-containing material aligned with the calyx/polyhedron envelope. Photo courtesy of G. Williams. From [169], with permission.

Polyhedron associated proteins

Ac68 may be involved in polyhedron morphogenesis. Homologs of Ac68 appear to be present in all baculoviruses. When Ac68 was deleted, no major effects were detected other than a longer lethal time in larvae [18]. Similar results were obtained when the ortholog in BmNPV (Bm56) was deleted from a bacmid. However, the polyhedra produced by the mutant BmNPV bacmid were abnormal and lacked virions suggesting that Bm56 may be involved in polyhedron morphogenesis [19].

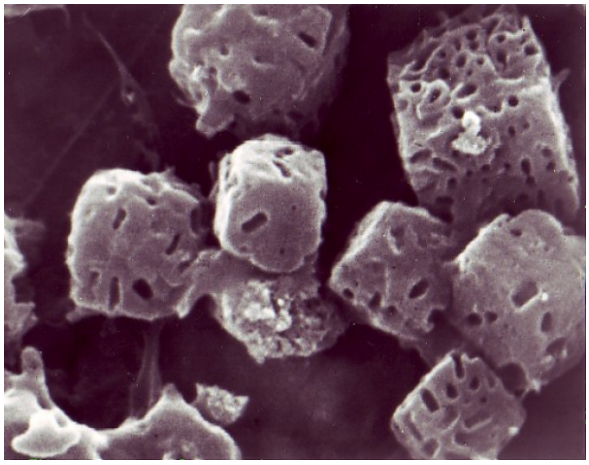

Fig. 2.5. Polyhedra from OpMNPV with the polyhedron envelope protein and p10 genes deleted. From [13], with permission.

P10 (Ac137). Although p10 does not appear to be a major occlusion body protein, it colocalizes with the PE protein and appears to be required for the proper formation of the polyhedron envelope. When p10 is phosphorylated, it becomes associated with microtubules [20]. This could be related to the structures it forms that include microtubule-associated filaments, and tube-like structures that surround the nuclei of infected cells [21, 22]. Deletion of P10 results in polyhedra that resemble those produced by mutants lacking the calyx/polyhedron envelope protein; they are fragile, have a rough surface showing cavities where virions have apparently become dislodged, and often show an incomplete calyx/polyhedron envelope [13, 23, 24] (see Fig. 2.5). There are a number of features of p10-deleted virus that are likely to be secondary effects. These include reduced cell lysis [25] and an increase in infectious virus [23]. Homologs of p10 are found in the genomes of all Group I/II NPVs and most GVs, in some instances in multiple copies, e.g. PlxyGV [26]. Their phylogeny is complicated somewhat by the fact that some of the GV genes appear to consist of a combination of p10 and polyhedron envelope protein domains [27, 28]. A p10 homolog has also been characterized in an entomopox virus [29].

Viral enhancing factors: enhancin. Enhancins are a class of metalloproteinases [30] that are

encoded by a few lepidopteran NPVs (e.g., Ld-, Cf-, and MacoNPV) and GVs (e.g. Ag-, As-, Tn-, XcGVs). In one study of TnGV, enhancin was estimated to comprise up to 5% of the mass of occlusion bodies [31]. In another virus, LdMNPV, it was found to be associated with ODV envelopes [32]. Enhancin genes are often present in multiple copies, e.g. the XecnGV genome has four copies [33]. In LdMNPV which encodes two enhancins, deletion of either results in a 2- to 3-fold reduction in potency, whereas deletion of both caused a 12-fold reduction [34]. Enhancin is thought to facilitate baculovirus infection by digesting the peritrophic membrane (PM). The PM forms a barrier in insect guts that prevents the ready access of pathogens to the epithelial cells. The PM is rich in chitin and intestinal mucin protein and enhancins appear to target the degradation of the mucin thereby facilitating access of virions to the underlying cells [35].

Enhancins shows sequence homology with high levels of significance (e.g. 3e-29) to predicted proteins of a number of pathogenic bacteria, e.g., *Clostridium botulinum*, and a variety of *Bacillus* (e.g., *B. anthracis*) and *Yersinia* (e.g., *Y. pestis*) species. To investigate their function, enhancin homologs from *B. cereus*, *Y. pseudotuberculosis*, or TnGV were cloned into a construct of AcMNPV that yielded occluded viruses. Although the LD50 of these constructs was found to be about half of wt, only the construct expressing the TnGV enhancin caused a reduction in survival time. In addition, the bacterial enhancins failed to degrade insect intestinal mucin. It was suggested that the bacterial enhancins may have evolved an activity distinct from their viral homologs [36].

Proteinases. The initial research on occlusion body structure was hindered by the presence of proteinases that degraded the proteins under investigation. These preparations were derived from insect carcasses in various states of disintegration and decay. In hindsight, it is not surprising that these preparations showed proteinase activity. The finding that the proteinases could be heat inactivated [37], led to the ability to purify and eventually sequence a number of polyhedrin proteins before the advent of DNA sequencing technology [38, 39]. Subsequently it was found that the proteinases associated with occlusion bodies had properties similar to enzymes associated with the insect gut [40] and that polyhedra produced in cell culture lacked associated proteinases [41-43]. Although many baculoviruses encode a cathepsin-like proteinase, in AcMNPV (Ac127) it was most active under acidic (pH 5) conditions [44]. Therefore, the proteinases associated with occlusion bodies are likely a combination of enzymes derived from bacteria, the insect gut, and the virus.

Baculovirus envelope proteins

For AcMNPV and other relatively well-characterized lepidopteran NPVs, there are two types of virions produced during the virus infection; in insects the infection is initiated by occlusion-derived virus (ODV) that are released into the insect midgut upon dissolution of the occlusion bodies. ODV initiate infection in the midgut epithelium and the virus propagated in these cells are budded virus (BV) that exit the cells in the direction of the basement membrane and spread the infection throughout the insect. Late in the infection, virions become occluded within the nuclei of infected cells and are released into the environment upon the death and disintegration of the insect. The major difference between BV and ODV is the origin of their envelopes. BVs derive their envelopes as they bud through the host cell plasma membrane that has been modified by viral proteins. In contrast, ODV obtain their envelope in the nucleus and it may be derived from nuclear membranes that are modified with a number of viral proteins. Whereas, viral contributions to the BV envelope may be limited to one or two proteins, ODV envelopes are very complex. They appear to contain a number of virally encoded proteins, and in some instances it is difficult to separate them from capsid proteins.

Envelope proteins of budded virions

Probably the most well characterized baculovirus structural protein is the envelope fusion protein of Group I baculoviruses, GP64, because a relatively simple assay for its fusion activity was developed early in its investigation [45]. Because of early studies elucidating the importance of this protein for BV infectivity [46, 47], it was unexpected when genome sequence analyses of a variety of baculoviruses revealed that many of them lacked homologs of the *gp64* gene e.g. [48, 49]. Furthermore, it was determined that these viruses use a different fusion protein called F and that homologs of F are retained in *gp64*-

containing viruses (Fig. 2.6). These F homologs in viruses encoding GP64 are inactive as fusion proteins suggesting that *gp64* was obtained by a baculovirus and displaced the fusion function of the F protein, but the F gene was retained. Although both GP64 and F are low-pH activated fusion proteins, F appears to belong to a lineage of fusion proteins present in a variety of viruses, whereas *gp64* belongs to a different group (reviewed in Ref. [50]). These proteins are described below.

GP64, (Ac128). GP64 is a fatty acid acylated glycoprotein [51] and a low pH activated envelope fusion protein [52-54] that is one of the three most abundant proteins found associated with AcMNPV budded virions [55]. It is a member of class III viral envelope fusion that are characterized by the lack of a requirement for cleavage in order to be activated. Other members of class III are found in the Rhabdoviridae and Herpesviridae. They are characterized by an internal fusion domain, a stem domain proximal to their intercalation into the membrane, and a carboxyl terminal anchor domain [56]. The presence of the *gp64* gene is one of the major distinguishing features of the Group I viruses. Deletion of AcMNPV *gp64* is lethal and results in viruses that replicate in a single cell, but cannot bud out and infect surrounding cells [47, 57]. It is thought that all Group I viruses use GP64 for the entry of BV into cells, whereas all other baculoviruses lack a *gp64* homolog and appear to use the F (Ac23 homolog) protein as their envelope fusion protein except for hymenopteran NPVs, which lack both genes (Fig. 2.6). In addition to the Group I NPVs, orthologs of *gp64* are also found in thogotoviruses, which are members of the Orthomyxoviridae [58].

The postfusion structure of AcMNPV GP64 indicates that six of seven disulfide bonds are conserved between thogotovirus and baculovirus GP64 and one forms an intermolecular bond involved in trimer formation [59]. The data indicates that the fusion peptide and receptor binding sites co-localize to a hydrophobic patch at the tip of the trimer. It was suggested that transient forms of GP64 embed hydrophobic side chains into cell membranes triggering endocytosis independent of specific receptor molecules. This lack of specific receptors and the affinity of GP64 for cell membranes may explain its ability to facilitate the entry of AcMNPV into a wide variety of different cell types. In addition, it was suggested that the lack of a high affinity receptor is consistent with AcMNPV systemic infections. Since the infection has already been initiated by midgut infection using a mechanism independent of GP64, GP64 has evolved to spread the infection to as many cell types as possible via systemic infection. A comparison of the major structural features of GP64 and F proteins is shown in Fig. 2.7.

Mutational analysis of gp64 disulfide bonds indicated that whereas the intermolecular bond between Cys24 and Cys372 was not essential for membrane fusion or budding, viable virus that incorporated the mutations could not be generated. In contrast, mutational analysis of the 6 conserved intramolecular disulfide bonds indicated that all were essential for mediating membrane fusion [60].

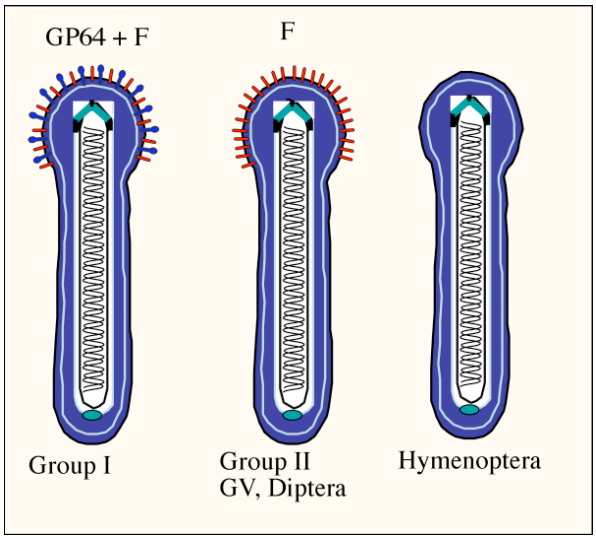

Fig. 2.6. Distribution of envelope fusion proteins. Group I have homologs of both GP64 and F, but F is not a fusion protein. Group II, GVs and dipteran viruses have homologs of F, whereas the hymenopteran viruses have homologs of neither GP64 nor F.

Evolution of Group I (GP64 containing) baculoviruses. As indicated in Chapter I, Group I baculoviruses appear to have been a lineage that had, in addition to gp64, 11 genes not present in other lepidopteran NPVs. Since many groups of NPVs have sets of genes not present in other lineages, this would not have been extraordinary as such genes might be lost and gained over time. However, the incorporation of

gp64 appears to have changed the biology of the lineage enough so that they evolved into a distinct group of viruses [61]. In addition, this evolutionary event was recently recapitulated with the demonstration that *gp64* could pseudotype and partially rescue a Group I virus deleted for its F protein [62]. However, in that experiment, the recombinant virus lacked both the F protein and the other genes unique to the Group I lineage. The latter include genes encoding the transactivator IE-2 (Ac151) and the global transactivator ortholog among others that could have facilitated the evolution of this lineage after it incorporated *gp64* [61].

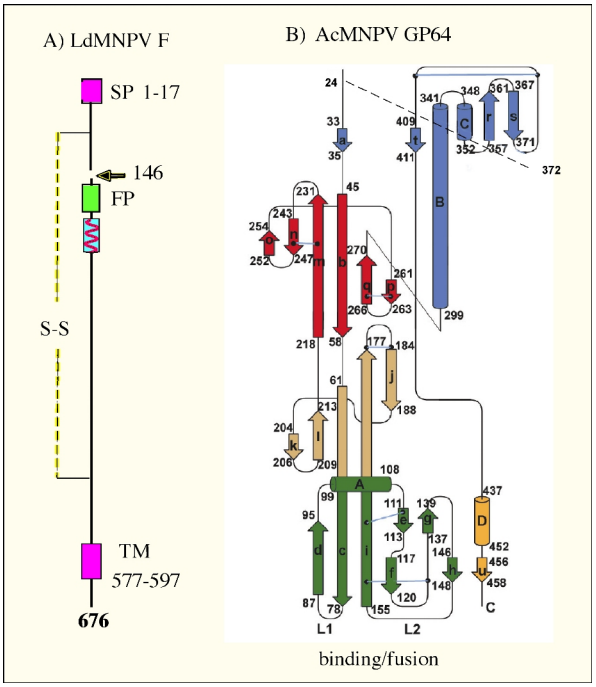

Fig. 2.7. Structure of the baculovirus F (fusion) protein (Ld130) from LdMNPV and AcMNPV GP64. A) Ld130 F protein. Shown is a predicted signal peptide (SP), fusion peptide (FP) and transmembrane domain (TM) including the amino acid coordinates. The cleavage site is indicated by the arrow. A predicted coiled coil domain is also indicated. The disulfide bond is predicted from [170]. B) GP64. This figure is derived from Kadlec [59]. Shown are 7 disulfide bonds all of which are intramolecular except for the one from aa 24 which is connected to aa 372 in an adjacent molecule. This and the coiled-coil (299-341) region are involved in trimer formation. All the disulfide bonds are conserved except 178-184 which is not present in thogotovirus GP64. The receptor binding/fusion peptide region is shown at the base of the diagram. From Kadlec et al [59]. Copyright 2008 by Nature Publishing Group. Reproduced with permission of Nature Publishing Group via Copyright Clearance Center.

Fusion protein-F, (Ac23). Although F (Ac23) is not an active envelope fusion protein in AcMNPV, in Group II NPVs, GVs, and the dipteran virus (CuniNPV) orthologs of F are likely used as the fusion protein, because all these viruses lack homologs of *gp64*. An exception appears to be the hymenopteran NPVs that lack homologs of both the F and GP64 proteins (Fig. 2.6) (see discussion in Chapter 3). F proteins of Group II NPVs function as low-pH envelope fusion proteins [63] [64] and can also rescue AcMNPV lacking *gp64* [65]. The F protein of granuloviruses has been implicated as a fusion protein due to their lack of a *gp64* ortholog. Although the gene encoding one such protein (PlxyGV orf26) failed to mediate fusion when incorporated into the genome of an AcMNPV mutant bacmid lacking *gp64* [65], another from *Agrotis segetum* GV (Agse orf108) was able to rescue AcMNPV lacking *gp64* [66]. F proteins appear to be members of a large and diverse family of viral envelope fusion proteins called Class I reviewed in [59]. They are present as homotrimers and are synthesized as a precursor that is cleaved by a furin-like proteinase into two subunits and near the amino terminus of C-terminal peptide is a hydrophobic fusion peptide (Fig. 2.7). Class I fusion proteins include many other viral fusion proteins such as influenza HA (e.g., see [67, 68]). Recent evidence suggests that the F and GP64 categories of fusion proteins may be distantly related [59].

Although inactive as a fusion protein in Group I viruses, the F protein homolog in OpMNPV (Op21) is glycosylated and associated with the envelope of BV and with the membranes of OpMNPV infected cells [69]. In AcMNPV, the F homolog (Ac23) is also associated with BV membranes and its deletion from the genome results in infectious virus with titers similar to wt in cultured cells, but the time to kill larvae is somewhat extended [70]. In addition, antibodies against some selected regions of GP64 appeared to inhibit binding of BV to Sf9 cells of Ac23 deleted virus to a greater extent than wt virus. This was interpreted to suggest that Ac23 may increase the binding of BV to Sf9 receptor molecules [71]. It could also indicate that Ac23 is closely associated with GP64 in the virion

envelope and when it is absent, the antibodies have greater access to GP64. Two reports have described investigations in cultured cells on AcMNPV in which *gp64* was substituted with the *F* gene from SeMNPV (Se8). In one study a construct with Ac23 was not completely rescued by Se8 [65], whereas in the other, the presence of Ac23 appeared to be required for the elevation of infectivity to near wt levels [72]. It was suggested that these contrasting results might be due to the different strength of the promoters used to express Se8 in the two constructs [72]. In another study, AcMNPV deleted for Ac23 produced more singly enveloped nucleocapsids [73]. Proteomic studies found Ac23 to be associated with AcMNPV ODV as was the homolog in CuniNPV [74, 75]. This suggests that it is transported to the nuclei of infected cells. The significance of this is not clear. Homologs of the baculovirus F gene are also found as the env gene of insect retroviruses (reviewed in [50]) and are also present in some insect genomes [76, 77] (see Chapter 11).

Ac16, (BV/ODV-E26). Evidence for the association of Ac16 with BV and ODV envelopes has been reported for AcMNPV [78] [74] [55]. However, in BmNPV, the homolog of AcMNPV Ac16 (Bm8) was not identified as a virion structural protein [79]. It was suggested that these conflicting results were due to the different sources of the antibodies used to detect the protein [80]. Homologs of Ac16 are found in lepidopteran Group I NPVs. Ac16 interacts with FP25 (Ac61), forms a complex with cellular actin [78], and is palmitoylated [81]. A mutant in which AcMNPV orf16 (called DA26) was insertionally inactivated was viable and showed no difference from wt in infections of *T. ni* or *S. frugiperda* cells or larvae [82]. In another report a virus deleted for Ac16 infected cells showed a delay in BV production [83]. It has also been shown to interact with both IE-1 and IE-0 and may be involved in the regulation of these gene products [84].

The cell receptors for budded virions – still searching. Evidence indicates that GP64 is the receptor binding protein of AcMNPV [85]. It is also well documented that GP64 can mediate the entry of AcMNPV into a wide variety of vertebrate cell lines (e.g., [86]). However, the identification of the cell receptor for budded virions has remained elusive. In one study, it was suggested that cell surface phospholipids might be involved in the AcMNPV BV (GP64 mediated) entry into vertebrate cells since treating cells with phospholipase C reduced reporter gene expression in cells [87]. In addition, acidic phospholipids in giant unilamellar vesicles are required for fusion with AcMNPV BV envelopes [88]. Other reports indicate that BV of a virus that used an F fusion protein did not have the ability to enter the array of vertebrate cells as AcMNPV. Consequently, it was suggested that the F fusion proteins might use a different receptor from GP64 [89] [90]. In contrast, another report described the ability of UV-inactivated viruses to compete for receptors and suggested that they have similar modes of entry [62].

Envelope proteins of occlusion-derived virus

The source and content of the envelope is the major distinguishing feature between BV and ODV. In contrast to BV, where one or two virus-encoded proteins have been identified as envelope associated, the ODV envelope is much more complex (Fig. 2.8). There may be five or more such proteins categorized as envelope proteins and another set of proteins called per os infectivity factors [91] that are likely envelope components. Some of these proteins contain an N-terminal hydrophobic sequence in combination with several adjacent positively charged amino acids. These have been predicted to be motifs that target these proteins to intranuclear microvesicles that are the likely precursors from which the envelopes of occluded virus are derived [80]. The following proteins (see also Table 2.2) have been characterized and are likely to be components of ODV envelopes (see also Fig. 2.8).

BV/ODV-E26, (Ac16), see above.

ODV-E66, (Ac46). ODV-E66 is a component of ODV envelopes [92]. When the N-terminal 23 amino acids of ODV-E66 are fused to a reporter gene, it is targeted to the nucleus [93]. Such a sequence would be necessary since the protein must localize to the nucleus for ODV morphogenesis. Evidence suggests that Ac46 encodes an enzyme, hyaluronan lyase, that is capable of digesting hyaluronan, a polysaccharide that is a major component of the extracellular matrix [94]. The extracellular matrix is a tissue component that provides structural support for cells. Homologs of this gene are

found in all Group I NPV, GV and most Group II NPV genomes, but not in hymenopteran or dipteran viruses. When the homolog of ODV-E66 was inactivated in BmNPV (Bm37), the mutant, although viable, took more time to kill insect larvae [95]. The homolog of Ac46 from *Helicoverpa armigera* NPV with PIF-2 and PIF-3 [96].

Table 2.2. Occlusion Derived Virus Envelope Proteins and Per os infectivity factors

AcMNPV orf # and name	**Distribution in the Baculoviridae**	**Effect of Deletion**
ODV envelope proteins		
Ac16, BV/ODV-E26	Lep. I	Viable [82]
Ac46, ODV-E66	Lep. I, II, GV	Viable [95]
Ac94, ODV-E25	Lep. I, II, GV	Not viable [100]
Ac109, ODV-EC43	All	Not viable [171] [102]
Ac143 ODV-E18	Lep. I, II	Not viable [104]
Per os infectivity factors		
Ac22, pif-2	All	Viable (not per os) [110]
Ac96, pif-4	All	Viable (not per os) [111]
Ac115, pif-3	All	Viable (not per os) [110]
Ac119, pif-1	All	Viable (not per os) [110]
Ac138, p74	All	Viable (not per os) [108]
Ac145	All but CuniNPV	Viable (reduced per os) [117]
Ac148, ODV-E56 pif-5	All	Viable (not per os) [106]
Ac150	Lep. I (a few)	Viable [117]

ODV-E25, (Ac94). The protein encoded by this gene was originally identified in OpMNPV, and immunogold staining with a specific antibody showed that it was localized to ODV envelopes [97]. It has also been shown to be associated with ODV of AcMNPV and *H. armigera* NPV (HearNPV) by proteomic analysis [74, 98] and with AcMNPV BV [55]. Similar to ODV-E66, the hydrophobic N-terminal 24 aa of AcMNPV ODV-E25 appears to contain a nuclear targeting signal [93]. Homologs are present in all lepidopteran NPV and GV genomes, but not in those of hymenopteran or dipteran NPVs. Deletion is probably lethal as mutant virus deleted for this gene in OpMNPV [99] or BmNPV [100] could not be isolated.

ODV-EC43, (Ac109). Homologs of Ac109 are present in all baculovirus genomes. Evidence suggests that it is ODV-associated in AcMNPV [74] and HearNPV (Ha94-ODV-EC43 [98, 101]. Although deletion of Ac109 did not appear to affect DNA replication or the appearance of BV, the virions were non infectious [102].

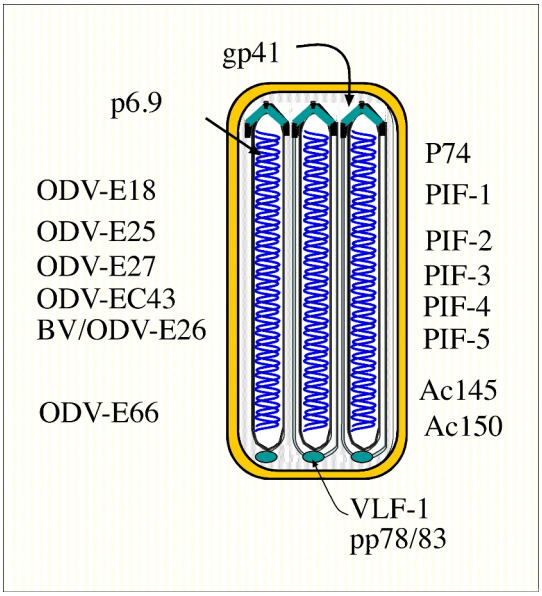

Fig. 2.8. Selected structural proteins of ODV. Shown are envelope associated proteins (ODV-E), the PIF proteins (also envelope associated), the tegument protein, gp41, the DNA binding protein, p6.9, and two basal end-associated proteins, pp78/83 and VLF-1. There are a variety of other capsid proteins (see text), but they appear to have a more generalized distribution. For details see the text.

ODV-E18, (Ac143). An antibody generated against an Ac143-GST fusion reacted with a protein of 18 kDa in the ODV envelope fraction and Ac143 was named ODV-E18 [103]. Ac143 and its HearNPV homolog were found in surveys of ODV-associated proteins by proteomic analysis [74, 98]. Homologs of Ac143 are present in all baculoviruses and it is an essential gene [104]. Deletion of the ODV-E18 gene results in single cell infections that produce polyhedra and therefore appears to be essential for BV

production [104]. In addition to its presence in ODV envelopes, it is also BV associated [55].

ODV-E56, PIF-5 (Ac148). ODV-E56 localizes to the envelopes of occluded virions [105]. Ac148 and its homologs in HearNPV and CuniNPV were found to be ODV associated [74, 75, 98]. Homologs of ODV-E56 are present in the genomes of all baculovirus genomes. ODV-E56 has been shown to be a per os infectivity factor required for midgut infection but not for infection infection to tissue culture cells [106] [107] (for more information, see the next section).

Ac144 (ODV-EC27) See below.

Per os infectivity factors (pif genes); additional envelope proteins

Per os infectivity factors were originally identified because they were required for infection of insects, but dispensable for infection of cultured cells [91]. Six pif genes have been identified in AcMNPV. They include, *p74-pif* (Ac138) [108], Ac 22 (*pif-2*) [109], Ac115 (*pif-3*) [110], Ac96 (*pif-4*) [111], Ac119 (*pif-1*) [91], Ac148 (ODV-E56, *pif-5*) [106] [107] (Fig. 2.8). Orthologs of these genes are present in all baculovirus genomes and are also found in genomes of nudiviruses [112] and some are found in other virus pathogenic for invertebrates (see Chapter 1). AcMNPV mutants lacking *pif 1*, *2*, or *3* are not orally infectious for *T. ni* or *S. exigua* larvae based on feeding of 10,000 PIB of the deleted virus. They are also not orally infectious for *H. virescens*, except for the PIF2 mutant that shows limited infectivity. In contrast, intra haemocoelic injection of 1 pfu of the three deletion mutants into third instar larvae of these three insect species caused over 80% mortality. In addition, PIF1, PIF2, and p74 mediate specific binding of occlusion derived virus to midgut cells, suggesting that they are directly involved in virus cell interaction as an initial step in infection [110]. Although PIF-3 appears to be an ODV associated protein [113], it does not appear to be involved in specific binding and its function is not known. P74 [114] and a homolog of PIF-1 from *Spodoptera littoralis* NPV [91] have been shown to likely be components of ODV envelopes. Subsequently it was found that AcMNPV PIF1, PIF2, and PIF3 are part of stable complexes located on the surface of virions and that p74 also might be associated with this complex [96]. Interaction of PIF proteins was also observed for *H. armigera* NPV using yeast 2 hybrid analysis. Several PIF proteins also interacted with HaNPV homologs of Ac98 (38K) and Ac46 (ODV-E66) [96]. A 35-kDa binding partner for AcMNPV P74 was detected in extracts of brush border membrane vesicles from host larvae (*S. exigua*), but not from a non-host (*H. armigera* larvae) [115]. The identity of this host protein has not been determined. By proteomic analysis, p74 was found associated with AcMNPV, HearNPV and CuniNPV ODV, [74, 75, 98]. However, the other PIF proteins showed variable ODV associations; e.g., CuniNPV ODV were associated with PIF-1,2,3; AcMNPV ODV with PIF-2; and HearNPV with PIF-1 and ODV-E56 (PIF-5). Ac145 and Ac150 also may be *pif* genes (see below).

Ac 145 and150, (more possible *pif* genes). These two genes encode small proteins (~9 and 11 kDa, respectively) that are related to one another (23% amino acid sequence identity). Close relatives of Ac 145 are found in all baculoviruses except the dipteran NPV. In contrast to Ac145, close relatives of Ac150 are only found in a few NPVs closely related to AcMNPV. Ac145 and 150 are predicted to encode a domain thought to bind to chitin [116]. In one study [117], deletion of AcMPNV Ac145 led to a six fold drop in infectivity in *T. ni,* but not *H. virescens* larvae. An effect of deletion of AcMNPV Ac150 was not detected. Deletion of both genes causes a major (39 fold) reduction of infectivity for *H. virescens*. Injection of BV of the double mutant intrahemocoelically produced the same level of infection as injected wt BV, suggesting that these genes play a role in oral infection. Ac145 and150 were found to be associated with both BV and ODV [117]. In another study [118], occluded virions deleted for Ac150 were found to be significantly less virulent when administered per os than the wt virus in *Heliothis virescens*, *Spodoptera exigua* and *Trichoplusia ni* larvae. Evidence suggested that the mutant had a reduction in its ability to establish primary infections in midgut cells.

Enhancin. In LdMNPV evidence suggests that enhancin is associated with ODV envelopes [32]. See above.

Nucleocapsid Structure

Several methods have been used to investigate nucleocapsid-associated proteins including identification using monospecific antibodies and mass spectrometry of purified peptides. A major problem with investigating structural proteins is to determine whether a protein found in association with virions means that it is an actual integral structural component, or is fortuitously present because it functions in close proximity to the location of virion assembly and becomes non-specifically trapped during virion assembly. This may be particularly true when proteomic analysis is used to identify ODV associated proteins. Whereas these investigations confirm the presence of many proteins previously implicated as structural components, they also identify many more that would not be predicted to be part of the virion structure [74, 75, 98]. These include a variety of proteins involved in DNA replication and transcription. It is unknown whether the presence of these proteins is adventitious or if they are bona fide structural proteins and play a role in accelerating the initiation of the infection cycle. The presence of proteins, such as DNA polymerase likely reflects an intimate relationship between DNA replication and packaging, and nucleocapsid assembly and envelopment. In addition, lipids can be 'sticky' and proteins in close proximity during virion assembly could adhere to the envelope and co-purify with ODV. Evidence from proteomic analysis of BV support this theory as they lack proteins associated with DNA replication and transcription suggesting that they were stripped off the nucleocapsids as they moved through the cell to the cytoplasmic membrane [55]. Furthermore, the facultative association of proteins with BV was demonstrated when it was found that BV can trap baculovirus expressed chloramphenicol acetyl transferase [119]. Likewise, some proteins that are present in polyhedra and associated with occluded virions may have a lesser affinity for the envelope and may be lost during the ODV purification process.

The advent of the use of bacmid knockout constructs to examine virion structural proteins has improved this situation somewhat. However, a defect in virion structure determined by this approach could be due to a secondary effect on some other structure. An example is the DNA binding protein DBP. It is not associated with virions, but when deleted, virions appear to be structurally defective. Since DBP associates with the virogenic stroma and when deleted, this structure is absent, it has been suggested that the role of DBP in virion structure is caused by the contributions of DBP to the structure of the virogenic stroma. Without a properly formed virogenic stroma, the assembly of virions is aberrant [120].

BV and ODV Nucleocapsid associated proteins encoded by all baculoviruses

In addition to the proteins described above, there are some proteins that are likely to be present in both BV and ODV nucleocapsids (Table 2.3).

P6.9, (Ac100). P6.9 is a DNA binding protein and one of the three most abundant proteins found in proteomic analysis of BV [55]. It is a small (55 aa) arginine/serine/threonine rich protein [121]. Homologs appear to be encoded by all baculovirus genomes, but may be difficult to identify in computer analyses because of their small size and repetitive amino acid content. P6.9 was originally identified as a DNA binding protein in a GV [122] and the homolog was subsequently identified from AcMNPV [121]. The high concentration of arg and ser/thr residues is similar to protamines that are present in sperm nuclei of many higher eukaryotes and are involved in the production of highly condensed DNA. Protamines are also small molecules of 44-65 amino acids [123, 124]. Arginine has a high affinity for the phosphate backbone of DNA and the polyarginine tracts in protamines neutralize the phosphodiester backbone, whereas the ser and thr residues interact with other protamine molecules thereby yielding a neutral, highly compact DNA-protein complex that is biochemically inert. P6.9 localizes to the nuclear matrix during infection [125]. Once viral DNA has been delivered to the nucleus, p6.9 is phosphorylated resulting in both DNA and p6.9 being negatively charged. It is thought that this causes the removal of p6.9 from the viral DNA thereby allowing access to transcription factors [126]. AcMNPV has two candidate kinases that could be involved in this process. Protein kinase 1, PK1 (Ac10), is encoded by lepidopteran baculoviruses (GVs and NPVs), whereas PK2 (Ac123) is encoded by only a few baculoviruses closely related to AcMNPV. Neither protein was found to be associated with ODV by mass spectrometry [74, 75, 98]. However, a kinase was found associated with BV and ODV, although its source was not determined [127].

P6.9 is not required for DNA replication, however viruses deleted for the gene are not viable and form tube-like structures similar to those produced by the VLF-1 knockout [128].

Table 2.3 Selected Proteins Associated with Baculovirus Nucleocapsids

Name and AcMNPV orf #	Distribution in the Baculoviridae	Effect of Deletion or mutation
Ac100, P6.9 DNA binding	All	Not viable [128]
Ac89, VP39	All	Not viable [100]
Ac80 GP41 tegument	All	Not viable [137]
Ac98 38K	All	Not viable [138]
Ac142	All	Not viable [144, 146]
Ac144	All	Not viable [144]
Ac66	All	Severely compromised [160]
Ac92 (P33)	All	Not viable [172] [173]
Ac54 (VP1054)	All	Not viable [137]
Ac77 VLF-1	All	Not viable [174]
Ac104, VP80	Lep. I and II NPV	Not viable [100]
Ac9, PP78/83	Lep. I and II NPV	Not viable[157]
Ac129, P24	Lep. I, II, GV	Viable [167]

VLF-1, (Ac77). The very late factor, VLF-1, is a member of the lambda integrase (Int) family of proteins and was originally identified because it influences the hyperexpression of very late genes [129] possibly by binding to their regulatory regions [130]. Whereas mutations to the region that affected very late gene transcription were not lethal, other mutations, including mutation of a conserved tyrosine that is required for integrase activity, inactivated the virus [131]. VLF-1 appears to be a structural protein present in both BV and ODV [131] and is clearly required for the production of nucleocapsids. Bacmid deletion mutants produce tube-like structures that stained with vp39 antiserum suggesting that the lack of VLF1 prevents normal capsid assembly. Bacmids with point mutations in the conserved tyrosine form normal appearing capsids, but are also not infectious. VLF-1 localized to the end regions of nucleocapsids further suggesting that it is a structural protein [132]. VLF-1 was also associated with ODV virions as determined by proteomic analysis in AcMNPV and CuniNPV, but not HearNPV [74, 75, 98]. For additional information, see Chapter 5.

VP39, (Ac89). VP39 is thought to be the major capsid protein. It is one of the three most abundant proteins found by proteomic analysis of BV [55]. Homologs of vp39 are present in all baculovirus genomes. It was originally characterized in OpMNPV [133] and AcMNPV [134].

GP41, tegument protein, (Ac80). GP41 is modified with O-linked N-acetylglucosamine, and is located between the virion envelope and capsid in a structure called the tegument [135, 136]. Homologs are present in all baculovirus genomes. Based on the characterization of a ts mutant, it is an essential gene required for the egress of nucleocapsids from the nucleus [137]. Under the restrictive temperature, this mutant produces no BV and infection is limited to single cells.

Ac98 (38K). Ac98 is encoded by all baculoviruses and is associated with both BV and ODV nucleocapsids. By yeast two-hybrid assays it interacted with VP1054, VP39, VP80, and itself [18]. When deleted, tubelike structures devoid of DNA but that stain with vp39 antibody are produced [138]. It appears to have homology to CTD phosphatases by the HHpred program [139].

Ac141 (exon0). Ac141 is conserved in all lepidopteran baculoviruses and is associated with both BV and ODV nucleocapsids [140] [55] and interacts with BV/ODV-C42 (Ac101) and FP25 (Ac61) [141]. It appears to be required for the efficient transport of nucleocapsids from nuclei through the cytoplasm [140, 142]. It appears to both co-localize with and co-purifiy with β-tubulin. In addition, inhibitors of microtubules reduced BV production by over 85%. Therefore it has been suggested that the interaction of Ac141 with microtubules might be important in the egress of BV [143].

Ac142, (p49). Ac142 is associated with both BV and ODV virions. Deletion of Ac142 appeared to

affect nucleocapsid formation but, although lethal, did not appear to affect DNA synthesis [144]. Similar results were reported for the homolog in BmNPV (Bm118) [145]. Another study describing a different bacmid construct that deleted less of the Ac142 gene showed similar results except that nucleocapsids, although fully formed, were not enveloped in nuclei and were not occluded [146]. It is unclear whether the difference in the two studies was due to the removal of a 3' processing signal for the upstream Ac141 gene in the former investigation, or to the presence of a significant portion of the Ac142 orf in the latter study. Proteomic analysis also suggests that Ac142 is ODV-associated in three different viruses [74, 75, 98]. Homologs have been identified in all sequenced baculovirus genomes.

Ac144. *Ac144* encodes a virion-associated protein. It was suggested that Ac144 is a multi-functional cyclin and may be involved in regulating the cell cycle during virus infection [147]. It was reported to interact in a yeast two-hybrid assay with Ac101 (also named C42, see below) and both Ac101 and p78/83 in native gel electrophoresis assays [148]. Although this orf was designated as ODV-EC27 (i.e. a 27 kDa protein of ODV envelopes and capsids) [103], another investigation found that it is more similar in size (33.5 kDa) to that predicted from the sequence and that it was associated with capsids, but not envelopes of BV [144]. It was also found that deletion of Ac144 resulted in defective nucleocapsids and although lethal, did not appear to affect DNA synthesis [144]. Proteomic analysis suggests that Ac144 is ODV-associated in three different viruses [74, 75, 98]. Homologs have been identified in all sequenced baculovirus genomes.

Other structural proteins

The following structural proteins are found in some, but not all baculovirus genomes. This could indicate either that they are not present or have evolved to such an extent that their relatedness can no longer be identified in the genomes in which they are not found.

VP80, (Ac104). Homologs of Ac104 are found in all Group I and II lepidopteran NPV genomes, but not in GVs or hymenopteran or dipteran NPVs. The homologous orf in OpMNPV encodes an 87 kDa capsid-associated protein of both BV and ODV [149]. Evidence suggests that it is also capsid associated in AcMNPV [150] [55] and CfMNPV [151]. It is an essential gene in BmNPV; deletion mutants of Bm88 appear to replicate in single cells indicating that there may be a defect in BV production preventing spread of the virus [152].

PP78/83, (Ac9). PP78/83 is a phosphorylated protein that is located at one end of nucleocapsids [153, 154]. It is a Wiskott-Aldrich syndrome protein (WASP)-like protein. Such proteins are involved in nuclear actin assembly and it has been demonstrated that pp78/83 serves this function during AcMNPV infection [155] [156] (see Chapter 3). Homologs of pp78/83 are found in all lepidopteran NPV genomes. It is an essential gene and because it is located adjacent to the polyhedrin gene, it was originally manipulated via complementation to elevate the frequency of obtaining recombinant baculoviruses at the polyhedrin locus [157].

BV/ODV-C42, (Ac101). Ac101 encodes a capsid-associated protein. Homologs have been identified in all sequenced baculovirus genomes with the exception of the virus pathogenic for the dipteran, *Culex nigripalpus* (CuNiNPV) [7]. It is associated with BV and ODV [74]. In addition, it was reported to interact in a yeast two-hybrid assay and by native gel electrophoresis [148] with phosphoprotein 78/83 (pp78/83) (Ac9) (described above) and is required for its transport into nuclei [158]. Deletion of Ac101 affected nucleocapsid formation but, although lethal, did not appear to affect DNA synthesis [144].

Proteomic analysis of budded virions

Analysis of BV from AcMNPV using mass spectrometry identified 48 virally encoded proteins and 11 host proteins [55]. Of viral encoded proteins, the envelope fusion protein, GP64, the major capsid protein, VP39, and the DNA binding protein p6.9 were found in the most abundance. 35 proteins previously reported to be associated with BV were identified along with an additional 13 proteins including PP31, AC58/59, AC66, IAP-2, AC73, AC74, AC114, AC124, chitinase, polyhedron envelope protein

[159], AC132, ODV-E18, and ODV-E56, previously not reported to be associated with BV. In contrast to proteomic analysis of ODV, no proteins associated with DNA replication or transcription were detected in the analyses of BV suggesting that they are trapped during ODV assembly because of the close association of transcription and DNA replication when virions are produced. Of the 11 host proteins, cytoplasmic actin and actin depolymerizing factor were found perhaps reflecting the role actin plays in BV transport [156].

Proteins associated with ODV that are also likely to be components of BV

Ac66. Ac66 shows a high degree of homology to a variety of proteins including a predicted orf in the protozoan *Trichomonas vaginalis* (E=6e-20), an actin binding protein in *Dictyostelium discoideum* (E=1e-14), rabbit myosin heavy chain (E=2e-14), and centromere protein E of *Canis familiaris* (E=2e-14). It has a number of conserved domains including one related to Smc (structural maintenance of chromosome), which is found in chromosome segregation ATPases that are involved in cell division and chromosome partitioning. It also shows homology to desmoplakin, a component of desmosomes that are structures that hold cells together (also called adhesion junctions) and are the main adhesion junction in epithelia and cardiac muscle. It was found to be associated with AcMNPV and HearNPV (Ha66) ODV [74, 98]. Ac66 is oriented in the opposite direction of DNA polymerase (Ac65), and its promoter region overlaps with the 5' region of the DNA polymerase orf. This orientation is conserved in many, if not all baculoviruses. Although the orfs adjacent to DNA polymerase in *Neodiprion* (e.g. NeSeNPV) and CuniNPVs show little similarity to Ac66, they show homology to the same proteins as Ac66, e.g., a predicted orf in *Trichomonas vaginalis*. Therefore, it appears that homologs of Ac66 are present in all baculovirus genomes. An AcMNPV bacmid deleted for Ac66 was severely compromised and BV titers derived from transfected cells were reduced by over 99% compared to wt. In addition, at low titers the mutant BV appeared to infect single cells and was unable to spread to other cells. Although the nucleocapsids appeared to be normal and had an electron dense core suggesting that they contained DNA, they appeared to be trapped in the virogenic stroma suggesting that Ac66 was required for the efficient egress of virions from nuclei. The deletion did not affect the levels of DNA replication or polyhedrin transcription, but the production of occlusion bodies was eliminated [160]. This suggests that Ac66 is required both for egress of virions from nuclei and also may be involved in the nucleation of polyhedra.

Ac92, (P33). The *p33* gene appears to be conserved in all baculovirus genomes and encodes a sulfhydryl oxidase and is likely involved in the production of disulfide bonds in viral proteins [161]. It was originally of interest because it formed a stable complex with the tumor suppressor gene p53 when it was expressed in a baculovirus system and appears to enhance its apoptotic function [162]. Although orthologs of p53 have been identified in insect genomes, e.g., [163], it is unclear what role p33 might play in baculovirus infection. P33 was found to be associated with ODV virions by proteomic analysis in three different viruses [74, 75, 98]. Deletion of Ac93 is lethal (Wu and Passarelli, pers. Comm.).

VP1054, (Ac54). VP1054 was named for the size of its orf and encodes a protein required for nucleocapsid assembly. A ts mutant failed to produce nucleocapsids at the non-permissive temperature indicating that it is an essential gene. It is found in both BV and ODV [164]. Homologs are found in all baculoviruses.

P24-capsid, (Ac129). Ac129 (P24) was found to be associated with both BV and ODV of AcMNPV and OpMNPV by Western blot and electron microscopic analyses [165]. It is likely to be nonessential as interruption of this gene with a transposable element in a strain of AcMNPV has been reported [166, 167]. Homologs of Ac129 are present in all Group I /II and GV genomes. The Ac129 homolog was not reported to be associated with ODV of HearNPV [98]. It was also reported to be BV associated [55].

Ac145/Ac150, see above.

Other virion associated proteins: ODV associated proteins identified by proteomic analysis

Proteins associated with DNA replication and processing. A puzzling feature of the characterization of the lepidopteran ODV associated proteins by proteomic analysis has been the identification of several proteins that are involved in DNA replication and processing in these preparations. HearNPV preparations contained helicase, DNA polymerase, and LEF-3, the SSB that interacts with helicase and is required for DNA replication [98], whereas the study of AcMNPV ODV identified those proteins along with the viral PCNA homolog (Ac49), LEF-1 (Ac14, DNA primase), IE-1 (Ac147) and alkaline nuclease (Ac133). As described above, due to their absence in BV [55], these proteins are likely co-purified with ODV because of their close proximity to and involvement in the synthesis or assembly of ODV components and are trapped during envelope assembly or the occlusion process. They are likely stripped off as BV move through the cell. Consequently, they may not be integral components of the virion structure. It was found that BV can trap baculovirus expressed chloramphenicol acetyl transferase which confirms that random trapping of proteins occurs most likely because of their close association with virions during their assembly or transport [119].

Analysis of HearNPV ODV proteins by proteomic analysis identified orthologs of most of the proteins described above and most of these have been determined to be virion associated by other techniques in other viruses. In addition, two proteins that appeared to be specific to that virus (Ha100 and Ha44) were identified [98]. Forty-five virus-encoded proteins were found to be associated with ODV of the dipteran virus CuniNPV. Of these, 14 are described above and have been identified as virion associated using other techniques, homologs of 8 proteins from AcMNPV including the F protein (Ac23), BRO (Ac2), Ac68, Ac81, Ac92, Ac96, Ac98, LEF-9 (Ac62) were also identified. The other 23 proteins were specific to CuniNPV [75]. Analysis of AcMNPV identified 36 ODV associated proteins. Of these, 13 have been identified as being structural proteins using other techniques, 9 are associated with DNA replication or processing, and the others appear to be specific to AcMNPV [74].

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Chapter 3

The baculovirus replication cycle: Effects on cells and insects

The baculovirus replication cycle is complex and involves two types of virions; one that is occluded and is adapted for stability outside the host insect and for infection of midgut cells. In contrast, budded virions are not occluded and spread the infection from cell to cell within the insect. The major events in systemic infections are illustrated in Fig. 3.1.

To initiate an infection, the occluded virions must infect midgut cells and much of the evolution of this type of virion has focused on adaptations to this environment.

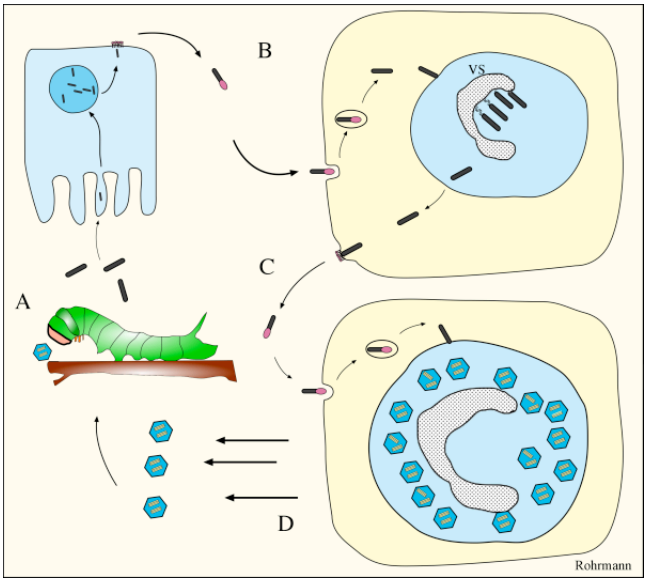

Fig. 3.1. A life cycle of a baculovirus causing systemic infection. Occlusion bodies ingested by an insect, dissolve in the midgut and ODV are released which then infect midgut epithelial cells (A). The virion buds out of the cell in a basal direction and initiate a systemic infection (B). Early in the systemic infection more BV are produced which spread the infection throughout the insect (C). Late in infection occluded virions are produced, and the cell then dies releasing the occlusion bodies (D). The virogenic stroma (VS) is indicated.

The insect midgut

To understand the baculovirus infection cycle, a brief overview of the insect gastrointestinal tract is necessary, since this is the site of the initial infection and several major features of baculovirus biology have evolved to accommodate and exploit this unique environment [1]. The insect gastrointestinal tract is composed of three sections, the fore-, mid- and hindgut. In Lepidoptera, the foregut is involved in facilitating the uptake, storage, and physical processing of food. It is lined with a chitin-containing cuticle that is part of the insect exoskeleton. A valve separates the foregut and midgut. The midgut is the major site of digestion of food and lacks a cuticle, but is lined with the peritrophic matrix (PM) (Fig. 3.2). The PM is composed of chitin, mucopolysaccharides, and proteins and it separates ingested vegetation from the midgut epithelium [2]. It is thought that it protects the gut surface from damage caused by abrasive food material and limits the access of microorganisms. It also allows the transfer of liquid and digested substances to the midgut epithelial cells, but prevents the passage of larger food particles. It is worn away by the passage of food and is regenerated from the epithelial cells. The most common midgut epithelial cells are columnar cells with a brush border that is adjacent to the gut lumen. Regenerative cells are present at the base of the epithelium and they replenish the columnar epithelial cells that become damaged and are sloughed into the lumen. Goblet cells are also present and may be involved in ion transport that regulates pH (Fig. 3.2). The midgut is involved in enzyme secretion and absorption of digested food and has a gradient of pH values. At the entry and exit of the midgut, the pH is near 7.0, but in the central region it can vary from 10.0 to as high as 12.0, depending on the lepidopteran species (Fig. 3.3) [3]. These are among the highest pH values found in biological systems. Another valve

separates the midgut and the hindgut. The hindgut is lined with a cuticle similar to the foregut and is involved in uptake of digested material, although to a lesser extent than the midgut.

Two types of virions

Baculoviruses have evolved to initiate infection in the insect midgut. This has led to two major features of baculoviruses; the environmentally stable but alkali-soluble occlusion body, and occluded virions that have an envelope and associated proteins that allow survival and infection in the harsh alkaline midgut environment that contains a variety of digestive enzymes. In contrast, the environment encountered within the insect has a near-neutral pH, and therefore is more benign. Upon release from occlusion bodies, the virions are called occlusion-derived virus (ODV) (see below). The other virus type, budded virions (BV) have an envelope distinct from ODV that facilitates systemic infection. These types of virions differ in their efficiencies of infection for different tissues; ODV infect midgut epithelial cells up to 10,000 fold more efficiently than BV, whereas conversely, BV are up to 1,000-fold more efficient at infecting cultured cells than ODV [4, 5].

From occlusion bodies to susceptible midgut cells

A common feature of the life cycle of all baculoviruses is the presence of virions embedded in occlusion bodies that are produced in the final stage of the replication cycle and are released upon the death and disintegration of the insect. Occlusion bodies serve to stabilize the virus in the environment and are normally only dissolved under alkaline conditions. High pH environments are rarely, if ever, encountered in most ecosystems and normally are only found in the midguts of some susceptible insects. Upon ingestion, the alkaline conditions of the midgut of larvae cause the dissolution of the occlusion bodies and the release of the ODV (Figs. 2.3, 3.2). The polyhedron envelope/calyx structure that surrounds the polyhedra is likely degraded by proteinases present in the gut or associated with the polyhedra (see Chapter 2). After their release from occlusion bodies, the first barrier that the viruses encounter in the insect midgut is

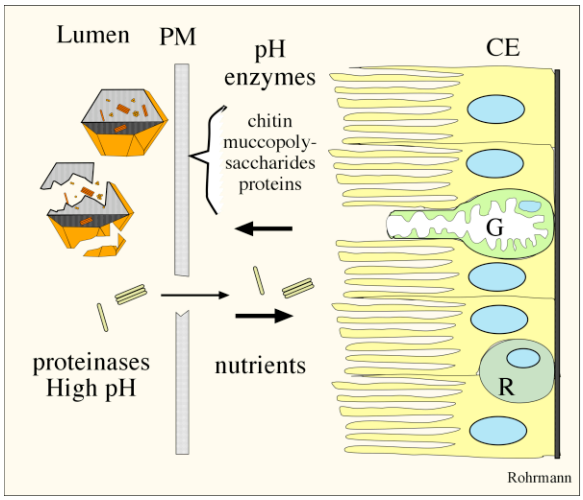

Fig. 3.2. The insect midgut and virus infection. The midgut cells generate the peritrophic matrix (PM) by the synthesis and secretion of chitin, muccopolysaccharides and proteins. They also secrete digestive enzymes and ions that regulate the pH. Occlusion bodies are dissolved by the high pH in the midgut lumen, and are further degraded by proteinases associated with occlusion bodies that may also digest the PM. The three major types of midgut cells are indicated, columnar epithelium (CE), goblet cells (G) and regenerative cells (R).

the peritrophic matrix (PM) (Fig. 3.2). As described in the previous chapter, polyhedra are nonspecifically contaminated by bacterial proteinases that function under alkaline conditions. Also some baculoviruses encode endopeptidases (metalloproteinases) that contain divalent cations as integral components of their structure. One category of metalloproteinases, called enhancins, is concentrated in occlusion bodies. It is thought that enhancins specifically digest mucin, a PM component, thereby allowing virus access to the epithelial cell surface [6]. Enhancins are only encoded by a limited number of baculoviruses and it is unclear how viruses that lack this enzyme pass through the PM. However, some are highly infectious (e.g., AcMNPV), and do not appear to be inhibited by the PM suggesting that there must be alternate or parallel mechanisms, such as the contaminating bacterial proteinases described above, that are involved in breeching the PM [7]. The following is an overview of the pathogenesis of AcMNPV and related viruses that cause systemic infections. These viruses, after initial replication in the midgut, spread throughout the organism. In

contrast, a variety of less well-characterized viruses limit their replication to midgut cells. At the end of this section, the pathogenesis of viruses that are confined to the midgut and do not cause systemic infections will be reviewed.

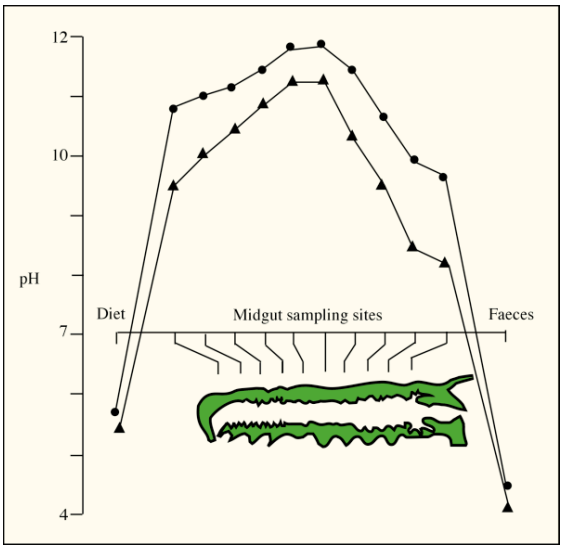

Fig. 3.3 PH profiles along the gut lumens of two lepidoperan species. The pH of the hemolymph was 6.7. The species shown are *Lichnoptera felina* (circles) and *Manduca sexta* (triangles). This figure is reproduced with permission of the Company of Biologists from [3].

Infection of midgut epithelial cells; per os infectivity factors [8] (see also Chapter 2). AcMNPV ODV normally initiate the replication cycle by infecting columnar epithelial cells that are a major cell type lining the midgut, and regenerative cells in *T. ni* [9] or *S. exigua* larvae [10] (Fig. 3.4). There are a combination of factors that appear to be involved in the initiation of midgut infections. These include factors that facilitate binding to the cells, cell receptors to which the virions bind, and virion envelope proteins that may have enzymatic activities that allow viral access to midgut cells or that fuse with the host cell membrane thereby permitting viral entry. A set of gene products called per os infectivity factors is required for infection of midgut cells (Fig. 3.4). They are specific to ODV and are not found associated with BV. They can be deleted from a viral genome without affecting the ability of the virus to infect cells in culture. However, such deletions reduce the ability of the virus to infect midgut epithelial cells. Six such genes have been identified including *p74-pif* (Ac138), Ac 22 (*pif-2*), Ac96 (*pif-4*), Ac115 (*pif-3*), Ac119 (*pif-1*), and Ac148 *(pif-5)*. Homologs of these six genes are conserved in all sequenced baculovirus genomes. This provides further evidence for a related pathway of infection for all members of this virus family. P74, PIF-1, PIF-2, PIF-4, and PIF-5 have been implicated as ODV envelope-associated proteins [12-16] and several have been shown to mediate specific binding of occlusion derived virus to midgut cells, suggesting that they are directly involved in virus-cell interaction as an initial step in infection [17] (Fig. 3.4). Although PIF-3 appears to be an ODV associated protein [18], it is apparently not involved in specific binding and its function is not known. PIF1, PIF2, and PIF3 appear to be part of stable complexes located on the surface of virions and p74 also might be associated with this complex [19]. It has been suggested that ODV binds to proteinase sensitive receptors [20] and once bound, the ODV envelope fuses with the epithelial cell membrane releasing the nucleocapsid into the cell cytoplasm.

Chitin binding proteins. Two other proteins, Ac145 and Ac150, have properties similar to PIF proteins and are described in Chapter 2. Both Ac145 and Ac150 are predicted to encode chitin binding domains [21]. In addition, Ac83 (VP91), a virion structural protein is also predicted to encode a chitin binding domain. There are two possible interactions involving chitin binding that might occur during midgut infection (Fig. 3.2). One would involve binding to the peritrophic matrix (PM) possibly to facilitate movement through the membrane. However, if the PM is extensively degraded during infection, this binding might be counterproductive. The chitin component of the PM is produced by chitin synthase, an enzyme that is located at the apical tips of brush border microvilli. The enzyme is also found associated with tracheal cells [22, 23]. Therefore, an affinity for chitin could facilitate the interaction of the virion with these cells, first to initiate infection of midgut cells (e.g., Ac145 and Ac150) and subsequently to interact with tracheal cells (VP91). Another protein, GP37 (Ac64) has been shown to bind to chitin [24]. Although GP37 has not been shown to be a structural protein, it may play a role in the infection of cells that synthesize chitin.

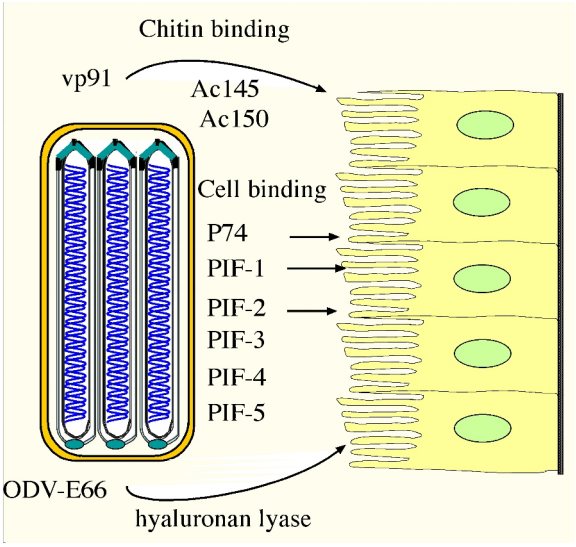

Fig. 3.4. Possible interactions of selected ODV envelope proteins and vp91 with midgut cells. VP91, Ac145 and Ac150 all have chitin binding domains suggesting that they may interact with chitin synthesizing cells. P74, PIF-1 and -2 bind to midgut cells. The role of PIF-3, 4 and 5 are not known. ODV-E66 has an enzymatic activity (haluronan lyase) that may assist in initiating the infection.

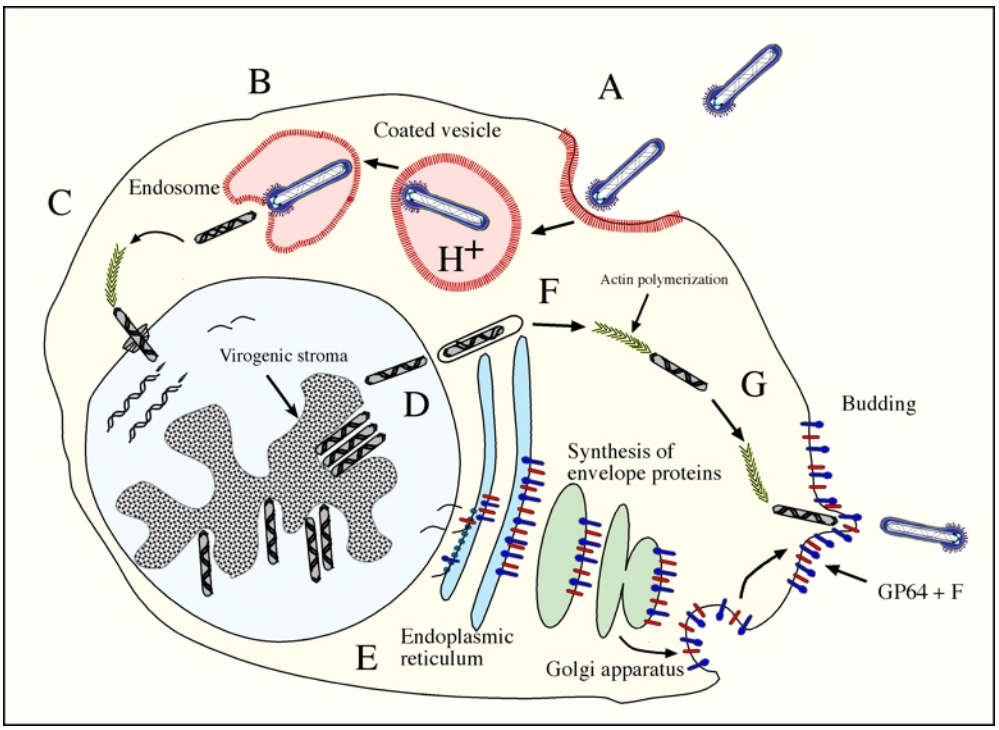

Fig. 3.5. Budded virus infection of a Group I virus. BV attach to receptors located in clathrin coated pits via GP64 and are endocytosed (A). The endocytic vesicle is acidified and this changes the conformation of GP64 and causes the virion envelope to fuse with the endosomal membrane releasing the nucleocapsid into the cytoplasm (B). The nucleocapsid enters the nucleus through a nuclear pore complex (C), genes are transcribed, DNA is replicated and nucleocapsids are assembled in the virogenic stroma (D). In Group I virus, at least two envelope proteins are synthesized, GP64 and F. They are likely translated in association with the endoplasmic reticulum, glycosylated and transported to and incorporated into the cytoplasmic membrane via the Golgi apparatus (E). Nucleocapsids destined to become BV exit the nucleus and are thought to transiently obtain an envelope that is lost (F). They move to the F and GP64 modified cytoplasmic membrane propelled via actin polymerization, bud through, and obtain envelopes (G).

ODV envelope proteins. Some ODV envelope proteins have been identified in addition to the PIF proteins. These are described in detail in Chapter 2 (Fig. 2.5) (also see Fig. 3.4). It is assumed that they facilitate fusion with the membrane of insect midgut cells [25]; however, the pathway used by ODV to enter these cells has not been delineated. One such protein ODV-E66 (Ac46) has hyaluronan lyase activity (Fig. 3.4) and is capable of digesting hyaluronan, a polysaccharide that is a major component of the extracellular matrix [26]. The extracellular matrix is a tissue component that provides structural support for cells. Therefore, this protein may facilitate access to midgut epithelial cells by digesting extracellular matrix tissue.

Entry into nuclei

After cell entry, NPV nucleocapsids are transported to the nuclear membrane in a process that involves actin polymerization [27, 28]. Several lines of evidence suggests that the nucleocapsids are then transported through nuclear pores (Fig. 3.5). Empty nucleocapsids were originally observed in nuclei of cells early in infection [25]. In human cells exposed to AcMNPV at high moi, at 4 hr p.i. about 8% of the nucleocapsids that entered cells were localized to the cytoplasmic side of nuclear pores. When mitosis was blocked, and the nuclear membrane was intact, nucleocapsids were observed in nuclei, suggesting that they do not need dividing cells and the corresponding nuclear membrane breakdown to enter nuclei. Because of their localization to the cytoplasmic side of nuclear pores and their presence in nuclei of cells treated with mitotic inhibitors which prevent the breakdown of the nuclear membrane that occurs during mitosis, these authors concluded that nucleocapsids can enter directly through nuclear pores [29]. Subsequently, using a virus with both a fluorescent tag fused to the vp39 capsid protein in addition to the wt vp39 protein, it was observed that the nucleocapsids localized to nuclear pore complexes and fluorescence was observed within nuclei [28] (Fig. 3.6). This suggests that the nucleocapsids dock with and are transported through the nuclear pore complex. Nuclear pore complexes have been recently characterized and have been calculated to have a channel of 38 - 78 nm [30]. Baculovirus virions have been calculated to have dimensions of 30-60 nm in diameter [31] indicating that they should be capable of moving through the pores. Once in the nucleus, the transcriptional cascade is initiated that eventually results in the production of nucleocapsids.

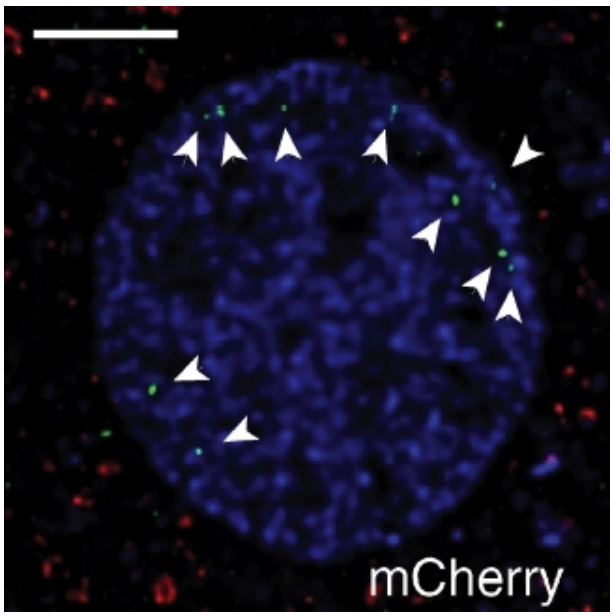

Fig. 3.6. Entry of AcMNPV nucleocapsids through nuclear pores. This shows virus with the capsid protein VP39 fused with the fluorescent label, mCherry. Arrows indicate capsids; bar is 5 μm. ©Ohkawa et al., 2010. Originally published in *J. Cell Biol*. 190:187-195.

Viruses that cause systemic infections (for virus infections restricted to the midgut, see below)

After nucleocapsids are replicated in the nucleus of midgut epithelial cells, to spread the infection, they need to exit the cell. It has been suggested that they bud out of the nucleus and in this process obtain an envelope from the nuclear membrane [25] (Fig. 3.5). The envelope may contain at least one viral protein, GP16 [32]. This envelope is lost during transit through the cytoplasm. Concentrations or patches of envelope proteins accumulate at the plasma membrane (Fig. 3.5). For group I NPVs, these include both GP64 (Ac128) and the F protein (Ac23) [33, 34] (See Fig. 3.5). In other viruses, e.g. members of Group II NPVs that lack *gp64*, this membrane is likely modified by homologs of the F protein [35]. The modification of the host

cell membrane by GP64 is required for virus budding and consequently for secondary infections [36, 37]. The distribution of GP64 in the initially infected midgut cells of *T. ni* larvae infected with AcMNPV is directed toward the basal and lateral regions of the cell [9]. This targets the virions to bud away from the gut lumen and toward other susceptible tissues, including neighboring cells [38]. This polar distribution of GP64 apparently does not occur in other tissues where targeting the infection in a specific direction would not be critical for spreading the infection [9].

Transiting the basal lamina. The initiation of systemic infections may differ based on the insect host. In particular, the pathway that viruses use to transit the basal lamina has been examined. The basal lamina is a structure composed of a fibrous matrix of glycoproteins that surrounds the midgut epithelium and is thought to be a barrier to systemic infection. Movement of AcMNPV has been examined using viruses expressing a *lacZ* reporter gene so the infection of single cells can be traced. In *T. ni* larvae, secondary infections appeared initially in tracheoblasts and tracheal epidermal cells. It has been reported that in some insects, tracheal cells have projections that penetrate through the basal lamina [39] (reviewed in [40]). Such projections could provide access to the tracheal system and allow the virions to move past the basal lamina leading to a systemic infection [38]. Further evidence for the ability of trachea to spread infections systemically was the observation that infections could be initiated by exposure of insects to BV through their spiracles. Such infections spread throughout the insect following tracheal tracts [41]. However, investigations of AcMNPV using a different insect, *S. exigua*, indicated that rather than the secondary infection specifically localizing to tracheoblasts, other tissues, such as midgut muscle, and hemocytes showed simultaneous infection suggesting that transit through the basal lamina might not have been dependent solely upon tracheoblast infection [10]. Surprisingly, it was noted in one of these studies that in most *T. ni* larvae, the midgut infection appeared to be transient and although, the infection had become systemic, it had been cleared from the midgut [38]. This was attributed to sloughing and regeneration of the midgut epithelium, possibly as a response to the infection.

Recently it has been suggested that a viral encoded ortholog of fibroblast growth factor (FGF) may be involved in movement of the virus across the basal lamina (see below).

Fibroblast growth factor (FGF), (Ac32) A possible factor in basal lamina transit. Ac32 has homology to fibroblast growth factor (*fgf*) and is termed vfgf. Homologs are found in the genomes of all lepidopteran baculoviruses (NPVs and GVs) and may reflect several independent lineages. AcMNPV *fgf* is closely related to a gene in *D. melanogaster* called *branchless*, whereas a Group II *fgf* ortholog from LdMNPV is less closely related to the insect homologs, and the GV orthologs show only limited similarity to NPV *fgf* orthologs. In BmNPV, FGF is glycosylated which is essential for its secretion [42, 43] and binds to an insect receptor called *breathless* [44]. AcMNPV FGF has been demonstrated to stimulate insect cell motility [45]. Although AcMNPV with a deletion of the *vfgf* gene showed no differences from wt in cultured cells [46], the time of death was delayed when it was fed to insects [47]. Similar results were observed for a BmNPV *vfgf* deletion [48]. Recent evidence suggests that vFGF initiates a cascade of events that may accelerate the establishment of systemic infections. This involves two processes. vFGF from virus infected midgut cells diffuse through the basal lamina and attract tracheal cells so that they are adjacent to infected midgut cells but separated by the basal lamina. vFGF then activates FGF receptors located on the tips of tracheal cells. This leads to the activation of matrix metalloproteases located in the same subcellular region via a MAP kinase or NFkB pathway. Matrix metalloproteases subsequently activate effector caspases that move extracellularly so that they are positioned for the degradation of the basal lamina by digestion of the laminin component. The delaminated tracheal cells are then susceptible to virus infection. This allows the transit of the virus through tracheal cells to other tissues and results in the systemic infection [49]. This theory is supported by evidence for the activation of matrix metalloproteinases, the activation of effector caspases, and the degradation of laminin after the per os infection of midgut cells.

Transiting midgut cells without replication. It has been suggested that under some circumstances nucleocapsids can transit through gut cells, bypassing replication, and bud directly into the hemolymph [25], or tracheal cells [50]. This may be a mechanism for accelerating

systemic infection and avoiding replication in gut cells that may be sloughed and eliminated from the insect. Genomes of all sequenced Group I viruses have both early and late promoter consensus sequences upstream of their *gp64* envelope fusion protein genes. Expression of *gp64* from the early promoter may prepare cell membranes for budding such that some nucleocapsids might directly transit the cell and bud without undergoing replication. When the early promoter was eliminated in the regulatory region of the AcMNPV *gp64* gene, a delay in the time course of infections initiated by oral, but not intrahemocelic inoculation of insects was observed [51, 52]. In addition, when *T. ni* cells are infected at high moi (200), many virions appear to become associated with the plasma membrane from within the cell [28]. This theory is applicable only to the MNPV type of virus, because it is dependent upon a cluster of connected nucleocapsids simultaneously infecting single cells, after which they would have to become separated in the cytoplasm with some entering the nucleus to undergo conventional replication. These replicating virions would direct the early synthesis of GP64 to prepare the cell membrane for budding of the nonreplicated nucleocapsids that transit directly through the cell possibly facilitated by the vFGF pathway described above. The theory is complicated by a lack of understanding of the MNPV type of virion morphology because it appears to lack a genetic determinant (see Chapter 1). A similar combination of early and late promoters is also present upstream of almost all of the F genes of Group II viruses suggesting that these two types of envelope fusion proteins (F and GP64) may be regulated in a similar manner. Whereas most of the Group I viruses are of the MNPV type (BmNPV may be an exception since single nucleocapsids predominate [53]), Group II viruses can be either MNPV or SNPV. For SNPVs, it is difficult to link the regulation of the fusion protein to the transit of the virus, because it would require extremely high levels of virus to ensure infection of single cells with more than one virion so that both transit and fusion protein synthesis could occur in the same cell. In contrast to the regulatory region of the F gene in Group II viruses, the regulatory region of the F gene orthologs in nine sequenced granulovirus genomes examined, lack conventional early promoter consensus sequences (TATA + CAGT) in the proper context and only 3 (AdorGV, AgseGV, CrleGV) have late promoter elements within 200 nt upstream of the ATG.

Secondary infection, cell entry (Figs. 3.1, 3.5). As described above, either GP64 or an active form of the F protein, is required for secondary infection. GP64 is an envelope fusion protein that, in addition to being required for exit from cells, is involved in initiating infection of other cells. It may also be an attachment factor, although the receptors it interacts with have not been defined. Since AcMNPV is capable of entering many types of cells, including those of vertebrates, receptors may be common molecules such as phospholipids [54]. In both insect and vertebrate cells, entry is mediated by GP64 via clathrin-mediated endocytosis [55] (Fig. 3.5). In this process, clathrin becomes concentrated in indentations or pits on the surface of plasma membranes. These structures are involved in the selective uptake of proteins into eukaryotic cells. Viruses commonly attach to receptors located on the surface of these clathrin-coated structures. The receptors contain internalization signals and upon binding to a viral attachment protein are stimulated to cause the pit to invaginate into the cytoplasm of the cell. This internalized vesicle is called an endosome or endocytic vesicle. It subsequently becomes acidified which causes the viral fusion protein to change conformation resulting in the merging of the viral envelope with the endosome membrane. This provides an opening or pore through which the nucleocapsid can enter the cell cytoplasm. It then moves to the nucleus and appear to enter through nuclear pores (see above) [25]. Once they have entered the nucleus, the DNA can be uncoated and the secondary replication can commence. It has also been demonstrated that AcMNPV can enter cells at low pH (pH 4.8) by apparently by fusing directly with the cell membrane independent of endocytosis [56]. However, it is not clear the cells and virus would encounter a pH this low under normal conditions.

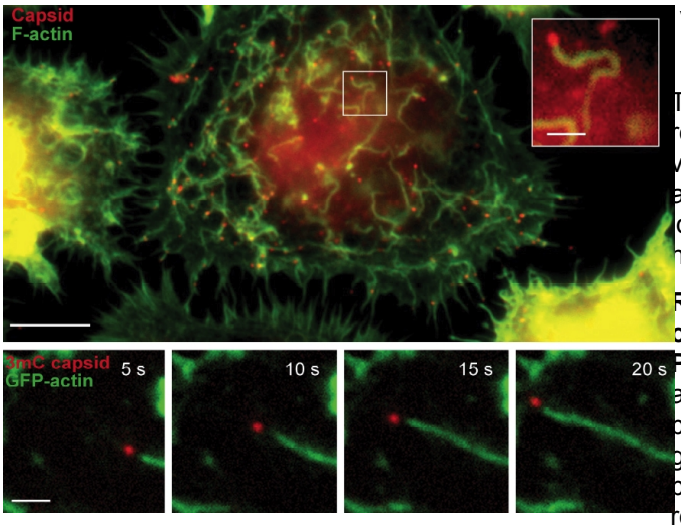

Fig. 3.7. Actin-based motility of AcMNPV within the cytoplasm of the cell. The virus was detected by capsid protein VP39 fused with the fluorescent label, mCherry (red) ; actin (green; FITC-phalloidin) Inset bar is 10 μm. Bottom: time series at 5 sec intervals. ©Ohkawa et al., 2010. Originally published in *J. Cell Biol*. 190:187-195.

The virogenic stroma. A novel structure characteristic of NPV- infected cells is the virogenic stroma (Figs. 3.1, 3.5). It is an electron dense, chromatin-like structure surrounding multiple less dense spaces that is found near the center of nuclei of infected cells. It is thought to be a molecular scaffold that is produced for the orderly and coordinated transcription and replication of viral DNA and the subsequent packaging of DNA and assembly of nucleocapsids. The structure of the virogenic stroma is not well-understood, but it appears to be composed of RNA and protein with discrete concentrations of DNA that border intrastromal spaces, the sites of virion assembly [57, 58]. In one study, it was observed that AcMNPV bacmids deleted for the single-stranded DNA binding protein, DBP (Ac25), failed to produce a virogenic stroma and also failed to produce normal-appearing nucleocapsids [59]. PP31 (Ac36) also appears to be associated with the virogenic stroma [60] and deletion of Ac36 results in a decrease in the level of transcription of some, if not all late genes [61]. Late in the infection virions may move from the virogenic stroma to a peripheral area where they become occluded [62], however, very late in infection, occlusion bodies can completely fill the nuclei. For a discussion of the virogenic stroma in relation to DNA replication, see Chapter 5.

Viral proteins involved in the infection cycle

The replication cycle described above is repeated resulting in the amplification and spread of the virus throughout the insect (Figs. 3.1, 3.5). There are some viral-encoded proteins that have been identified that facilitate this process. Several that have been characterized will be discussed here.

Reorganization of the cytoskeleton, the role of PP78/83 (Ac9) a WASP-like protein and P10. After AcMNPV infection of TN-368 cells, actin moves into nuclei and subsequently is polymerized from G- into F-actin. G-actin is a globular monomeric form of actin, and polymerizes into filamentous, or F-actin. This is a reversible reaction requiring ATP hydrolysis for polymerization. Eventually, during AcMNPV infection, most of the cellular actin is concentrated in nuclei. A cellular complex of up to 7 proteins is called the Arp2/3 complex. It is comprised of two actin related proteins (Arp) that resemble the structure of monomeric actin. This complex is involved in nucleating the formation of F-actin filaments. Activators are required for this process and they bind both monomeric G actin and the Arp2/3 complex. One category of such activators is called Wiskott-Aldrich syndrome protein (WASP) and an ortholog of WASP (PP78/83) (AcMNPV orf9) is encoded by all lepidopteran NPV genomes. A purified truncated version of AcMNPV PP78/83 containing the critical activation domains was found to be capable of stimulating actin polymerization in combination with the Arp2/3 complex in vitro. Studies of AcMNPV with mutations of PP78/83 suggested nuclear actin polymerization is required for the coordination of nucleocapsid development including the proper association of ODV with envelopes [63]. It was subsequently shown that nucleocapsids are propelled through the cytoplasm via actin polymerization that result in 'actin comet tails' trailing behind the nucleocapsid [28] (Fig. 3.7). In addition, a point mutation in the arp2/3 binding region of PP78/83 results in partially defective actin polymerization with reduced actin tails and erratic paths of movement that frequently changed direction. The use of actin polymerization for transport of intracellular pathogen have been observe for poxviruses [64] and certain bacteria, such as *Listeria monocytogenes* [65]. However, in contrast to these pathogens that employ actin polymerization after replication, AcMNPV uses it immediately upon infection before viral replication [28]. Evidence suggests that a capsid associated

protein, BV/ODV-C42 (Ac101), binds to PP78/83 and transports it into nuclei. Mutant bacmids lacking Ac101, fail to demonstrate polymerization of actin in nuclei [66].

A major feature of infection by lepidopteran NPVs is the massive reorganization of nuclei in which they expand to such an extent that they fill most of a cell's volume. It has been calculated that the diameter of Sf-9 cells may increase up to 1.45 fold during infection [67]. The movement and concentration of actin may contribute significantly to this key feature of these infections [63]. P10 also may contribute to cytoskeletal reorganization as it interacts with tubulin and may be involved in modifying microtubules [68, 69].

EGT, (Ac15). Another viral protein that can affect the course of an infection is an enzyme, ecdysteroid UDP-glucosyltransferase [70]. Egt homologs are found in all lepidopteran NPV (I and II) and all but two GV (XcGV and SpliGV) genomes, but not in other lineages (as of 2008). Because of its role in insect steroid metabolism, the likely source of a gene encoding this enzyme would be from a host insect, and closely related orthologs of *egt* are found in a variety of insects such as *B. mori*. The function of the viral EGT is to block molting and pupation in infected larvae by catalyzing the transfer of glucose from UDP-glucose to ecdysteroids, thereby inactivating these insect molting hormones [71, 72]. Molting can cause severe physiological stress on infected insects and many do not survive this transition. Therefore, the full productivity of the virus infection may not be realized. Evidence suggests that viral EGT prevents this stress by blocking molting. It also prolongs the feeding stage of infected larvae, thereby allowing the virus to replicate over a longer period of time in larger larvae, resulting in a higher yield of virus. The yield of occlusion bodies is increased about 30% in larva infected with wt virus compared to infection by virus lacking the *egt* gene [73, 74]. A remarkable feature of NPV infection is that in some instances the insects can grow and continue feeding right up until they die. They appear healthy, yet when examined, are heavily infected with high concentrations of occlusion bodies in their cells and hemolymph. EGT likely contributes to this effect. A common method to reduce the time that a virus takes to kill its host is to delete the *egt* gene. Larvae infected with these mutants are smaller and die sooner than wt, thereby reducing the damage caused to crops after the infection.

Budded versus cell-associated virus, is there a transition?

A major transition during baculovirus infections is the shift from BV production to the retention of nucleocapsids in the nucleus and their incorporation into occlusion bodies. In a study examining the kinetics of AcMNPV replication in cultured cells, the proportions of BV and cell-associated virus was analyzed using quantitative PCR to measure the number of viral genomes [75] (Table 3.1). The virus used in this study expressed *lacZ* in place of the polyhedrin gene and did not produce occlusion bodies It was found that viral DNA doubled every 1.7 hr starting at 6 hr postinfection until DNA replication ceased at about 20 hpi, which was correlated with the onset of budding. At this time point, under optimal conditions, virion-associated DNA reached a plateau at about 84,000 genomes per cell. In contrast, only about 2,000 genome equivalents per cell were released into the medium. This suggests that about 2.3% of the viral DNA is budded out of an infected cell. At the cell concentration employed (>3 × 10^6/ml), this would be equivalent to 6 × 10^9 BV/ml. Since baculovirus titers of about 5 × 10^8 pfu/ml are commonly achieved, this would suggest that about 10% of the BV were able to form plaques, a not unreasonable number. If these numbers reflect viral production in infected insects, then BV production might be a relatively minor component of the total virus produced. The possible inhibition of BV production late in infection could be the result of physical changes in the organization of the nucleus that limit nucleocapsid transit to the cytoplasm, or to the depletion of a component required for transit through the cytoplasm. It also is possible that as the infection progresses, ODV envelope proteins accumulate in the nucleus and once the nucleocapsids become enveloped they can no longer exit the nucleus. It has been reported that ODV-E25 (Ac94) localizes to the periphery of replication foci, whereas vp39 was found within these structures [76]. Therefore, early in the infection, naked nucleocapsids could transit into the cytoplasm, whereas later in the infection they might become enveloped and no longer be able

Table 3.1 DNA produced in AcMNPV infected cells (from [75])

Cells infected (moi = ~20)	AcMNPV genomes/cell	BV genomes/cell (% total genomes)	Host DNA (bp)/cell [77]	Viral DNA (bp)/cell
3×10^6/ml	84,000	2000 (2.3%)	$\sim 10^9$	$\sim 10^{10}$
5×10^6/ml	39,500	1750 (4.5%)	$\sim 10^9$	$\sim 5 \times 10^9$

to exit the nucleus. The numbers measured in the study by Rosinski et al. [75] also provide information on the viral DNA generated per cell during an infection. If for example, 84,000 genomes are produced per cell, this would be equivalent to 10^{10} bp/cell using 133,000 bp as the AcMNPV genome size. If the DNA content of a diploid *Bombyx mori* cell is $\sim 10^9$ bp [77], this would suggest that 10 times more viral DNA was generated during the infection than was present in the cell genome. Although this may seem high, it could be reflected in the expansion that nuclei undergo during the infection. Also, if much of this DNA is packaged into virions, it might be considerably more compact than cellular DNA. In addition, much of this DNA may remain unpackaged and this could be a factor underlying the ability of baculoviruses to hyperexpress late genes. Under these conditions, high levels of gene expression could be dependent on high gene copy number (see Chapter 5 for more detailed discussion).

Occlusion, the final stage in virus infection

As described above, most of the assembled nucleocapsids appear to be destined to remain in the nucleus and become occluded. Cell type seems to govern the production of occlusion bodies. For some viruses, midgut cells do not appear to support occlusion body production. However, for other viruses, this is the only locale where occlusion occurs (see below). The reason for this specificity is not clear.

In lepidopteran NPV infections, virions that remain in nuclei appear to obtain a membrane from microvesicles that may be derived from the inner nuclear membranes that have been modified with virally encoded ODV-specific envelope proteins [78]. A hypothetical model for this process is shown in Fig. 3.8. A feature of the final stage of baculovirus replication that takes place after most DNA replication has occurred is the hyperexpression of very late genes resulting in the production of high levels of polyhedrin and p10. Polyhedrin accumulates in nuclei and at some point crystallizes into a lattice that surrounds virions. It is not clear whether virion occlusion is a concentration-dependent, random event, or if virions serve to enucleate the formation of occlusion bodies. At least one

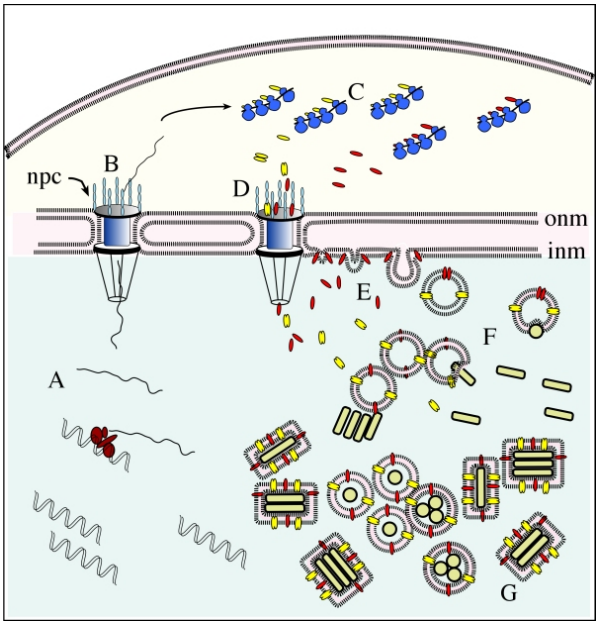

Fig. 3.8. A hypothetical diagram of ODV membrane morphogenesis. In this diagram mRNA encoding ODV envelope proteins are transcribed (A) and exported (B) to the cytoplasm for translation (C) and the proteins are then targeted to the nucleus (D). Some of these proteins may be targeted to the inner nuclear membrane and induce it to invaginate thereby forming microvesicles (E). The microvesicles may be further modified by the incorporation of additional virally encoded ODV envelope proteins and then virions become enveloped (F, G). Shown are the outer nuclear membrane [109], the inner nuclear membrane [110] and nuclear pore complexes (npc).

protein (Ac68) may be involved in this process because when the ortholog was deleted from BmNPV (Bm56), the virions produced were not incorporated into the occlusion bodies [79]. Also, very late in infection, high levels of P10 are expressed. It forms tube-like structures that penetrate both the nucleus and cytoplasm [69, 80]. As occlusion bodies mature, P10 fibrils align with the surface of the polyhedra and appear to be intimately involved with the assembly of the polyhedron envelope on the occlusion body surface (Fig. 2.2). The final polyhedron product has a smooth, even surface (Fig. 1.1). As discussed previously, if the polyhedron envelope gene is deleted the polyhedra produced have an uneven surface and virions appear to be prone to becoming dislodged (Fig. 2.3).

Virus dispersal

Many newly hatched lepidopteran larvae remain as concentrated populations near the egg mass from which they emerged. Dispersal is largely dependent upon access to food supplies that are rapidly consumed near the egg mass. However, during the final larval stages many Lepidoptera disperse (wander) probably as an evolutionary mechanism to spread the population, reduce predation, and also to find a suitable place to pupate. In *Manduca sexta,* wandering is induced by a limited rise in the ecdysteroid titer in the hemolymph, and is associated with the cessation of feeding and the commitment to begin pupation ([81] and references therein). Baculovirus infection appears to be capable of enhancing this behavior and can cause a terminally infected insect to migrate to a higher elevation on the branch of a tree or plant. This is thought to facilitate dispersal of the occlusion bodies. This condition was noted in Germany in the 1900s and named 'wipfelkrankeit' (tree-top disease) [82]. A gene (Ac1) encoding an RNA processing enzyme (RNA 5'-triphosphatase), has been implicated in the enhancement of this terminal movement of infected insects. Insects infected with BmNPV deleted for this gene failed to undergo the enhanced wandering behavior induced by wt viral infection [83]. How this enzyme can influence wandering behavior is unclear. Late in infection after the wandering stage, the insects become torpid and undergo what is termed 'melting'. Melting refers to the disintegration or liquifaction of the insect and is caused by some, but not all viruses. When on an even surface, the infected insects appear to flatten out and all the tissues appear to melt together and liquefy. Clearly, the disintegration of insects at a higher elevation on a plant and the subsequent contamination of lower vegetation could result in the infection of additional insect hosts, in contrast to simply dying, falling on the ground, and decaying into the soil.

Enzymes facilitating insect disintegration: Chitinase (Ac126) and cathepsin (Ac127). Some baculoviruses express enzymes that facilitate the disintegration of infected larvae late in infection. That an insect virus would obtain a chitinase gene to facilitate its dispersal might have been expected because chitin and chitinases are integral components of their host insects. The insect exoskeleton is composed of chitin, and because it is rigid, it must be periodically removed and reconstructed for insect larvae to grow. Therefore, insect larvae pass through various growth stages, or instars. The transition between these stages involves the digestion and absorption of part of the exoskeleton and shedding of the rest. Consequently, viruses infecting insects very likely had ready access to chitinase genes from their host insects. Chitinase genes are present in most lepidopteran Group I and II NPV and several GV genomes. The chitinase of these viruses is phylogenetically clustered with several lepidopteran chitinases, e.g., the AcMNPV (Ac126) and BmNPV (Bm103) proteins show over 60% aa sequence identity to *B. mori* chitinase. Although closely related, they have different properties; the AcMNPV chitinase is retained in the endoplasmic reticulum and functions under alkaline conditions, whereas the host enzyme is secreted and has reduced activity at higher pH [84-86]. In conjunction with another enzyme, a viral proteinase (cathepsin, Ac127, see below), chitinase participates in the liquefaction of insects late in infection. Insects infected with viruses in which either the chitinase or the cathepsin gene had been deleted remained intact for several days after death [87]. The retention of the viral chitinase in the ER may prevent the premature liquefaction of infected insects, allowing the virus to continue to replicate. The facility with which a virus (AgNPV) can be processed as a biocontrol agent for use against the soybean pest *Anticarsia gemmatalis* has been attributed to its lack of these two genes, thereby allowing collection of the virus from intact rather than disintegrated insects [88].

Orthologs of the viral cathepsins (Ac127) have a similar distribution to Ac126 (chitinase) and are present most Group I /II NPV and several GV genomes and they appear to work in concert. As with the viral and insect chitinase genes, the viral cathepsins are closely related to insect cathepsins, e.g., the Ac127 cathepsin is 39% identical to an Apis mellifera cathepsin. To further optimize the role of the viral cathepsin in insect liquifaction, it is synthesized in an inactive form that is activated upon death of the insect [89]. Therefore, both chitinase and cathepsin appear to have developed mechanisms to prevent their premature activation thereby prolonging the infection.

Because of the dramatic and gruesome nature of the final events in some baculovirus infections, it was incorporated into popular fiction by a science writer well before the current bioweapons frenzy. In this novel, a baculovirus is engineered to replicate in human brain cells and the resulting mayhem it causes when released into the human population is the story line [90].

The cytopathology of GVs

The above descriptions have focused on the replicative cycle of the most well characterized baculoviruses, namely NPVs of Lepidoptera that can be easily cultivated and genetically manipulated. However, there are a variety of other viruses that cause a more limited infection. Although some GVs have been investigated, understanding their cytopathology has been hindered by the lack of an efficient cell culture system. In one cell culture system that has been described, both infectious hemolymph and BV titers were low (the highest BV titer was 10^6 $TCID_{50}$/ml) and BV titers decreased with passage [91]. These low titers could reflect the insensitivity of the GV cell culture system and has made the generation of recombinant GVs challenging. Although GVs cause systemic infection, most GVs that have been characterized show a different pattern of cytopathology (Fig. 3.9) from the lepidopteran NPVs described above. In addition, it is difficult to generalize regarding pathology because of the variation of gene content in different types of GVs. For example, half the GV genomes characterized lack cathepsin/chitinase genes and some also lack EGT genes. After their initial replication in midgut cells, subsequent infections vary with different GVs (reviewed in [92, 93]). Some GV infections are limited to the midgut, whereas

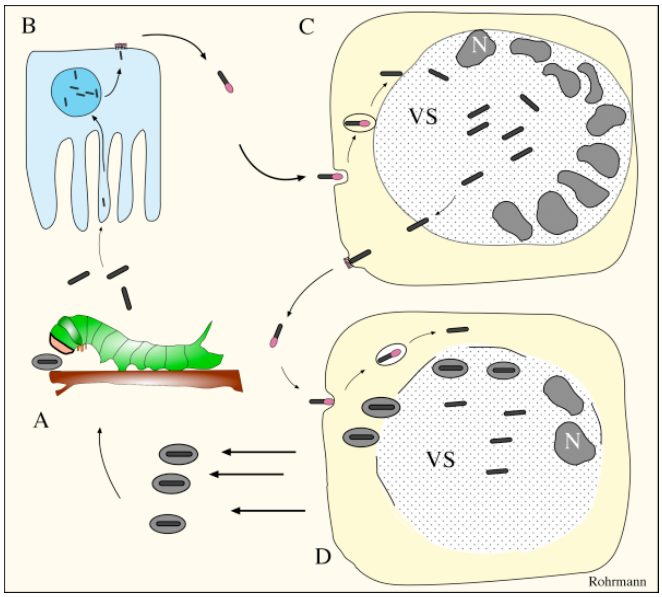

Fig. 3.9. A granulovirus life cycle with systemic infection. Many features of a systemic GV infection are likely to be similar to that of NPVs, including the infection of insect midgut and the systemic spread to other tissues (A, B). However, the GV infection leads to the clearing of the nucleus with nuclear material (N) locating to the margins (C) and the virogenic stroma (VS) distributed throughout the nucleus. Later in the infection the nuclear membrane becomes fragmented and the nuclear and cytoplasmic regions merge (D). This figure is interpreted from [91, 92].

others cause systemic infections and can replicate in a wide variety of tissues similar to NPVs. In addition, others appear to spread to and are limited to replicating only in fat body tissues. The cytopathology that occurs in the different cell types after GV infection, however, appears to be similar for all GVs and differs from NPVs (Fig. 3.9). In infected cultured cells, the nucleus enlarges and the interior becomes clear as electron dense material becomes concentrated at the periphery near the nuclear membrane. At this stage, nucleocapsids that are likely destined to develop into BV are evident in the nuclei, but occlusion bodies are not present. Later in the infection the nuclear membrane appears to disintegrate and the nucleoplasm and cytoplasm merge. After this point, occlusion bodies become evident [91]. Similar patterns of pathology have been found in infected insects

with some cells showing a well-developed virogenic stroma in nuclei bfore the disintegration of the nuclear membrane [92].

Viruses that are confined to the midgut; hymenopteran and dipteran NPVs

In some types of baculoviruses, the infection appears to be confined to the midgut. These viruses include NPVs of Diptera, e.g., mosquitos (CuniNPV) [94], Hymenoptera, and certain types of GVs mentioned above [95]. In the mosquito virus, CuniNPV, viral replication appears to proceed from an early production of virions that bud into the cytoplasm and the later development of occluded virions. The role of BV in spreading the infection to other cells is unclear [94]. The development of infections by NPVs of Hymenoptera is also not well characterized. Most surprising is the lack of a homolog of either gp64 or the F protein in the three hymenopteran NPV genomes that have been sequenced. Two candidates for genes encoding possible fusion proteins of 217 and 74 aa have been identified [96]. However fusion proteins of such a small size would be unprecedented for baculoviruses. Because of the requirement of fusion proteins for both BV cell egress and entry, it is unclear how infections by these viruses might spread. It is possible that infected cells are sloughed or disintegrate into the midgut lumen and release a mixture of occluded and nonoccluded virions containing an ODV envelope and other proteins that would allow infection of other midgut cells. In this case, ODV envelope and PIF proteins could facilitate this process. Genes predicted to encode homologs of these factors (including all the PIF proteins) have been identified in the genomes of the NPVs of Hymenoptera. These include *p74*, *pif-1,2,3, 4* and *odv-e18, -e27, -e56 (pif-5)*. A feature of at least some of these infections has been described as infectious diarrhea [93]. This could be a reflection of extensive cell death. Conserved proteinases that could be involved in this process are described below.

Possible dissemination strategies for GVs, and hymenopteran and dipteran NPVs. Although chitinase and cathepsin are found in almost all lepidopteran group I and II viruses, chitinase is present in only four and cathepsin in only three of the ten currently sequenced GV genomes (2008), and neither one is found in the genomes of the hymenopteran and dipteran viruses. However, there are other enzymes encoded in these viruses that might compensate for the lack of chitinase and cathepsin, especially when the infection is localized to the midgut. One such enzyme is a metalloproteinase (distinct from enhancin and cathepsin) with homologs present in all sequenced GV genomes. They have about 30% amino acid sequence identity to a catalytic domain in a stromelysin1 metalloproteinase of humans and sea urchins. The GV enzyme may be non-secreted and continuously active because it lacks both a signal peptide and a cysteine switch that maintains the homologous enzymes in an inactive form. The metalloproteinase from XcGV is capable of digesting proteins and is inhibited by metalloproteinase inhibitors [97]. The presence of metalloproteinase homologs in GV genomes may be involved in assisting in viral transmission by facilitating the disintegration of cells after the GV replicative cycle is complete. This might be reflected in infection associated diarrhea reported for some GVs [93]. Likewise, although hymenopteran viruses lack homologs of chitinase and cathepsin, they all encode a trypsin-like protein [98] that shows high levels of sequence identity (~50%) to insect trypsin-like orthologs. For baculovirus infections limited to gut tissues such as hymenopteran NPVs (see above), chitinase may not be necessary because chitin is not a major structural component of midgut cells. Therefore, the presence of a trypsin-like protein may facilitate the dispersal of virus from the gut cells, both by releasing the virus into the environment, and also by providing inocula for the infection of other gut cells.

Viruses with other tissue specificities. In contrast to viruses that are confined to replication in midgut cells, there has been one report of an NPV of a crane fly (*Tipula paludosa*) that specifically replicates in hemocytes [99] and the virus of the pink shrimp replicates in the hepatopancreas, an organ analogous to the vertebrate liver and insect fat body [100]. These viruses have not been further characterized.

Persistence/latent baculovirus infections

The possible presence of persistent baculoviruses has been suggested based on spontaneous outbreaks in controlled insect colonies, or by induction from exposing insects to factors such as cold stress [101]. Such occurrences have been reported for several different baculoviruses (reviewed in [102]). However, it was not until the advent of molecular biological technology including the polymerase chain reaction that methods became available to investigate these observations. The most carefully investigated recent description of a latent baculovirus infection has been reported for some, but not all, cultures of *Mamestra brassicae*, the cabbage moth. A virus, MbNPV, has been found to persist in most populations of *M. brassicae* in the United Kingdom [103]. The latent virus, can be activated when the persistently infected insects are fed either a closely related virus, *Panolis flammea* (pine beauty moth) NPV (PfNPV), or the more distantly related AcMNPV. PCR amplification identified MbNPV polyhedrin gene sequences in all insect stages including eggs, larvae, pupae, and adults. In the fourth instar larvae, the latent virus was found only in fat body tissue. A cell line was derived from this PCR-positive tissue that contained the latent virus [104] and it was estimated that the cell line contained 13-20 copies of the viral genome per cell [105]. In addition, it was found that virus-free *M. brassicae* larvae died of an MbNPV-like infection, when they were fed fat body cells from the latently infected laboratory strain of the insect. In another study, plasmids containing baculovirus late and very late promoters fused to the CAT gene were activated when transfected into latently infected cells. But when these plasmids were transfected into virus-free cells, such activation did not occur. This was interpreted to indicate that low levels of baculovirus genes were expressed in the latently infected cells and the proteins produced were able to activate the expression of the late promoter constructs [106].

In another investigation involving a GV of the Indian meal moth, *Plodia interpunctella*, it was found that transcripts of the PlinGV granulin were present in 60-80% of the offspring of insects that had survived exposure to a ~10% lethal dose of PlinGV. Inheritance of the virus was also observed in offspring of either exposed males or females mated with naïve insects [107].

It has been reported that persistent baculovirus infection can be caused by the infection of cells with AcMNPV that lacks the *p35* anti-apoptosis gene. These cell lines were resistant to subsequent challenge by AcMNPV infection and some of the cells contained high levels of viral DNA and exhibited early gene expression [108]. Under these conditions it would appear that there might be a balance between gene expression and the apoptotic pathway, and replication of viruses that lack *p35* selects for virus that do not induce apoptosis thereby allowing them to persist.

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Chapter 4

Early events in infection: Virus transcription

Transcriptional activators, enhancers and the host RNA polymerase

An underlying feature of gene expression in eukaryotic cells is that relatively few genes are concurrently transcribed and the genes that are expressed may change during development. Critical for regulating gene expression is a somewhat scarce set of proteins that comprise the transcriptional apparatus. The heart of this complex is an enzyme, RNA polymerase that synthesizes RNA. However, for RNA polymerase to selectively transcribe specific genes, a variety of transcription factors are required. These factors have different functions and can include: selecting the genes that will be transcribed; attracting and stabilizing the RNA polymerase complex; and facilitating RNA synthesis. Another category of critical factors are transcriptional activators that elevate the levels of specific RNA synthesis. These often function in conjunction with enhancers that are DNA sequences that bind transcription factors and bring them in close proximity to the RNA polymerase complex thereby elevating the levels of RNA synthesis. Collectively these DNA sequences, enzymes, and factors regulate transcription by influencing the rate at which RNA polymerase is recruited to, or stabilized, at a specific promoter and its subsequent ability to efficiently transcribe RNA. In higher eukaryotes, different transcriptional activators can cause the expression of ordered sets of genes at each stage of development, or can lead to the transcription of tissue-specific genes in differentiated tissues.

Viruses destined for productive infections are confronted with a dilemma upon entry into a cell: they must compete with the cell for control of its well-organized and programmed transcriptional machinery that is strictly regulated for carrying out cellular processes. Without special mechanisms to take control of the cell and focus its replicative machinery on the synthesis of viral products, the viral genome would languish in the cell and could be overwhelmed by competition from cellular genes. To outcompete the cell and focus transcription on the viral genome, viruses rely on several mechanisms. They can selectively inhibit host cell transcription or eliminate host mRNA. They also can encode their own set of transcriptional activators and enhancer sequences which facilitate the assembly of the host cell transcription complex at the site of RNA initiation of viral genes on the viral genome, rather than elsewhere, e.g., the host genome. Transcriptional activators can coordinate both the assembly of the transcription complex and by interaction with enhancer sequences, they can bring enhancer bound cellular transcription factors into close proximity to a gene promoter region. In some cases, this can elevate expression levels of viral genes several thousand fold, thereby allowing the virus to redirect cellular processes for its own replication. Many DNA viruses use this process to cause a transcriptional cascade upon infection. In this process, one of the first viral genes synthesized is a transcription factor that activates a set of genes including a second transcription factor. This second factor activates transcription of a second set of genes and so on until the transcription cascade is complete. This produces a regulated progression through a transcription program of categories of early and late genes that carry out the functions necessary for each stage of viral replication. In the case of herpes simplex virus, the transcriptional program is initiated by a transcriptional activator (vp16) that is a structural component of the virion and is transported into the nucleus during the initiation of infection. Other nuclear replicating viruses, e.g., adenoviruses, express a transcriptional activator early after infection, thereby initiating the transcriptional cascade. Throughout the transcriptional programs employed by these two viruses, the virus is dependent upon regulating the host RNA polymerase to transcribe its genes and thereby carry out its replication.

Baculovirus infection: selective effects on host cell gene expression

Early investigations using pulse labeling with ^{35}S methionine to examine protein synthesis in baculovirus infected cells indicated that as the infection proceeded, the host cell proteins faded and the viral proteins became more prominent [1]. This was subsequently correlated with the down regulation of host cell mRNA levels [2] [3]. It could also be a result of a phenomenon called translational arrest [4]. Although the precise mechanism is not known, since the virus is dependent on a variety of cell functions including RNA polymerase II, a generalized inhibition of all protein expression is unlikely in a permissive infection. More recent experiments have indicated that a number of host genes are up regulated during infection. For example certain mitogen activated protein kinases (MAPKs) appear to be required for BmNPV gene expression. Inhibition of the expression of some MAPK genes results in reduced virus production [5] suggesting that any inhibition of host gene expression that occurs is selective or that the virus is able to up regulate certain categories of host genes. Microarray analysis of BmNPV infect *B. mori* cells indicated that transcription of 37 genes was increased while expression of 17 decreased [6]. Using 2-D gel comparisons in combination with proteomic analysis a comparison of protein expression in cells permissive, semi- and non-permissive for AcMNPV infection was reported [7]. It has also been shown that baculovirus DNA replication can trigger both apoptosis and translational arrest thereby influencing the expression of host genes [8]. The conflicting evidence regarding levels of host protein synthesis could be due to the level of expression of the up-regulated genes. Many of the most abundant host proteins could be shut down, while other genes, particularly those with an essential enzymatic function, might be up regulated, but are not evident because their expression is relatively low. For additional discussion of the regulation of RNA polymerase II, see the section Shutoff of Early Transcription below.

The baculovirus transcription cascade: the evolution of a novel strategy

Baculoviruses begin their infection cycle similar to other large DNA viruses and employ enhancers and transcriptional activators to exploit the host transcriptional apparatus. This early program is focused on establishing the infection and producing the components necessary for initiating viral DNA replication and other early functions. However, whereas these early events are dependent on genes transcribed by the host RNA polymerase, the later genes are transcribed by a baculovirus encoded RNA polymerase (Fig. 4.1). Consequently, early in infection the transcription is carried out by the host RNA polymerase, whereas the viral RNA polymerase is involved late in infection. Although some bacterial viruses, e.g., T7, also exploit their host's RNA polymerase early and employ their own RNA polymerase later in infection, baculoviruses are the only nuclear replicating DNA viruses of eukaryotes that employ this combination of cellular and viral polymerases. Other eukaryotic DNA viruses that encode their own RNA polymerases such as pox viruses replicate in the cytoplasm and do not have ready access to the cellular transcriptional apparatus because of their separation from the nucleus. The evolutionary logic for baculoviruses to encode their own RNA polymerase is unclear. However, it may have originally been a major advantage for the virus to be able to regulate its own polymerase in the context of cells that were undergoing the shock of virus infection. This advantage could include the mitigation of possible effects caused by low levels of host RNA polymerase that might be normally present or caused by the infection (see Chapter 6).

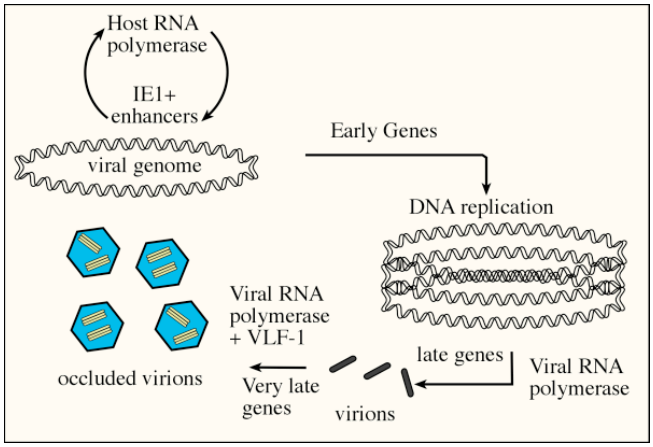

Fig. 4.1. The baculovirus transcriptional cascade showing the interrelationship of host and viral RNA polymerases and DNA replication and VLF-1.

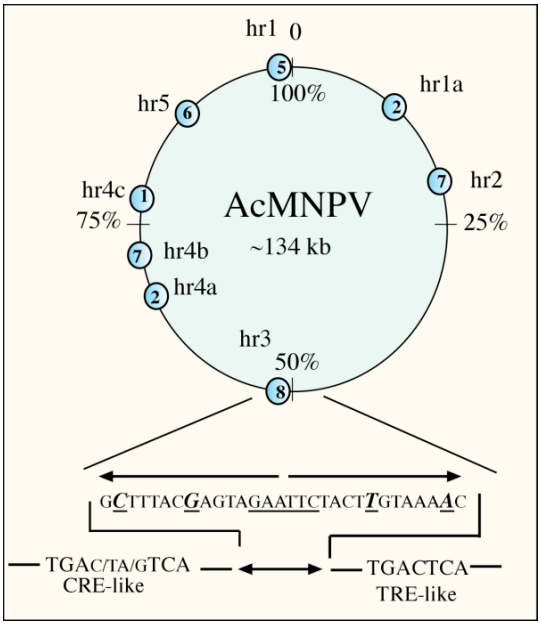

Fig. 4.2. Diagram of the AcMNPV genome showing *hrs*. The numbers in circles indicate the number of repeats in each *hr*. Below is shown a representative palindrome with mismatches shown as underlined italicized larger type. The EcoRI site at the center of the palindrome is also underlined.

Transcriptional enhancers

Similar to other viruses, enhancers and transcriptional activators play a major role in the early events in baculovirus infections. Enhancers are specific DNA sequences (Fig. 4.2) that are able to bind and concentrate transcription factors and can deliver them to the regulatory region of a gene, thereby facilitating transcriptional initiation (Fig. 4.3). They can be located near or at some distance from the site of transcriptional initiation. It is thought that, when distant, the enhancer DNA with its associated transcription factors can bend around and be positioned in close proximity to the site of transcriptional initiation (Fig 4.3). Bending of the DNA can be facilitated by transcriptional activators that bind to both the enhancer sequence, and to the RNA polymerase complex. In this process the RNA polymerase complex would bind to the gene promoter and the activator could then bind to the enhancer region and bring it and its associated transcription factors into close proximity to the RNA polymerase-promoter complex (Fig. 4.3). Whereas one might consider an enhancer sequence distant from a promoter, activators binding to the enhancer located on the same DNA molecule as a promoter would likely be in much closer proximity to the promoter than if they were floating free in the cell.

Baculovirus enhancers; *hrs* (homologous regions)

A characteristic feature of most baculovirus genomes is the presence of homologous regions, or *hrs*. In AcMNPV, *hrs* are comprised of repeated units of about 70-bp with an imperfect 30-bp palindrome near their center (Fig. 4.2). They are present at eight locations in the genome with 2 to 8 repeats at each site. They are highly variable, and although they are closely related within a genome, they may show very limited homology between different viruses. For example, in the CpGV genome, tandem repeated sequences are not evident, although a 75-bp imperfect palindrome is present at 13 different locations on the genome [9]. In addition, in the TnNPV (group II) genome, *hr*-like sequences were not found [10].

Hrs have been implicated both as transcriptional enhancers and origins of DNA replication for a number of baculoviruses [11-16]. In AcMNPV, they bind the transcriptional activator IE1 (Ac147) [17-19] and this binding can elevate the levels of IE1 transactivation up to 1000 fold [11]. In addition, *hr* binding may cause IE1 to localize to sites that may be a prelude to replication foci [20]. *Hrs* contain cAMP and 12-O-tetradecanoylphorbol 13-acetate (TPA) response elements (CRE and TRE)-like sequences located between the palindromes Fig. 4.2). A survey of baculovirus genomes found that these elements were preferentially concentrated within the *hr* sequences. In AcMNPV, although *hrs* comprise less than 3% of the total genome sequence, they contain 94% (48 of 51) of the CRE and 62% (13 of 21) of the TRE motifs. One to two of these elements are found between each pair of *hr* palindromes (Fig. 4.2). These are evolutionarily conserved throughout the lepidopteran NPVs, but are not found in GVs, or dipteran and hymenopteran NPVs. In other systems, these elements bind cellular transcription factors and stimulate RNA polymerase II dependent transcription. In AcMNPV, the CRE and TRE

were found to bind to host cell proteins and activate transcription in transient assays [21]. Despite the concentration of these motifs in *hrs*, their ability to activate transcription appears to be adapted to each virus, probably due to a reduction in the affinity of IE1 from one virus to the *hrs* of another. For example, AcMNPV IE1 is much more efficient in activating a reporter gene linked to an AcMNPV *hr* than is OpMNPV IE1 [22]. The consensus sequences of the *hr* palindromes between the two viruses are about 57% (17/30) identical [23].

It has been shown that deletion of individual or combinations of two *hrs* did not appear to affect AcMNPV replication in cultured cells [24]. This is probably due to the ability of transcriptional activators to interact with *hrs* in other locations of the genome allowing them to be brought into close proximity to the promoters being activated by bending of the DNA (Fig. 4.3).

A major transcriptional activator of early genes, immediate early gene 1 (IE1, Ac147)

To start the transcriptional cascade that initiates the baculovirus replication cycle, many baculoviruses employ the transcriptional activator, IE1. IE1 was originally identified because of its ability to transactivate early promoters of AcMNPV [25]. It is transcribed early in infection (hence the name: immediate early gene 1, *ie1*) and continues to be transcribed through the late phase [26]. Orthologs of IE1 have been identified in all Group I and II genomes sequenced. They also appear to be present in GV genomes, but the similarity is very low, e.g., XcGVorf9 and Ac147 are about 10% identical. However, the orientation and position of XcGV orf9 relative to more conserved orfs is similar to Ac147, suggests that Ac147 and XcGV orf9 are related. Part of the IE-1 population is called IE-0 and is translated from a larger spliced mRNA which is the only major spliced transcript described for baculoviruses (see below Ac147-0). In AcMNPV, either IE1 or IE0 can support viral replication (see below). AcMNPV IE1 contains two separate domains; an acidic activation domain located in the N-terminal region, and a DNA binding domain present in the C-terminal region [27, 28]. It also contains a dimerization domain that is associated with a predicted helix-loop-helix sequence near the C terminus [19, 29]. A positively charged domain adjacent to the dimerization domain is involved in nuclear import and is dependent on IE1 dimerization [30]. A conserved 10-aa basic region (aa 152-161-KIKLRPKYKK) separating two acidic activation domains was found to be required for DNA binding. It was suggested that this basic region neutralizes the adjacent acidic activation domains. In this model when the basic region interacts with DNA, the acidic domains are exposed allowing them to participate in activation [31]. IE1 from a related virus, OpMNPV, has activation domains similar to AcMNPV IE1 [32] and a region in the 65 N-terminal amino acids that is involved in both transcriptional activation and DNA replication. The replication domain was specific to the virus and other sequences that supported transactivation did not support replication in an OpMNPV transient assay system [33]. The ability of IE1 to transactivate transient transcription is greatly enhanced when the activated gene is linked to *hrs* [12]. IE1 may also participate in the negative regulation of some genes [34] (see below). IE1 is required for transient DNA replication [35, 36] and is an essential gene as a bacmid deleted for this gene was not viable [37]. Similar results have been reported for BmNPV [38].

Binding of IE1 to *hr* sequences

Although IE1 has been extensively investigated, purification of biologically active IE1 has not been reported. This suggests that it might be unstable and inactivated during purification procedures or that it is only active in combination with another protein(s). However, IE1 has been shown to bind to *hr* sequences independent of insect cell proteins. This was accomplished by transcribing the *ie1* gene in vitro, and then translating the resulting RNA in a rabbit reticulocyte system. The product was able to specifically bind to *hr* sequences indicating that additional insect cell proteins are not required for binding to occur [17, 39].

Many proteins, including type II restriction enzymes and numerous transcription factors, recognize and bind to palindrome sequences. Such proteins are normally found as homodimers and interact in such a way that the DNA binding site of each monomer recognizes the identical sequence, but on opposite sides of the palindrome. IE1 appears to follow this pattern in its interaction with the palindromes of *hr* sequences and both halves of a palindrome are

necessary for activation [19]. Footprinting analyses have not been straightforward, which may be due to the presence of two types of binding to DNA. If mixtures of these two types of binding occur, especially when mutants are being examined, the actual pattern of binding may be obscured. However, foot printing of wt sequences suggests that IE1 interacts with both halves of the palindrome, but on opposite DNA strands as described above. Although altering a palindrome half-site by mutation, prevents activation, IE1 still binds to the remaining site. However, the binding properties are changed, such that IE1 binds to both strands of the remaining half site [40]. It was suggested that this could explain the mechanism by which IE1 negatively regulated the expression of certain genes that have an *hr* half-site sequence located between their TATA promoter and mRNA start site motif [34]. By binding in this region, IE1 could prevent the binding and function of RNA polymerase II, thereby preventing transcription of the gene.

Despite their apparently critical role as transcriptional enhancers and probable role as origins of DNA replication, it is not clear how *hrs* are recognized by the proteins involved in these functions. It was suggested that they may form cruciform structures in the DNA that would provide landmarks for the binding of transcriptional activators. This has been observed in other palindrome-like structures, e.g., the high affinity binding of the cyclic AMP response element binding protein (CREB) to enkephalin enhancers [41]. However, studies in vitro were unable to detect the formation of cruciform-like structures by AcMNPV *hr*-like imperfect palindromes, although such structures were formed by perfect palindromes in which mismatches in the predicted *hr* structure were eliminated. In addition, IE1 was able to bind to both these structures [42].

Ac147-0 (ie-0): the only major spliced AcMNPV gene

Part of the IE-1 population is called IE-0 and is translated from a larger spliced mRNA which is the only major spliced transcript described for baculoviruses and in AcMNPV, results in an additional 54 amino acids at the N-terminus of IE-1 for a total of 636 amino acids [43]. Only one other example of a gene expressing a spliced mRNA has been identified among baculovirus transcripts. However, this mRNA, from OpMNPV, would be unlikely to produce a novel protein because of the location of a stop codon

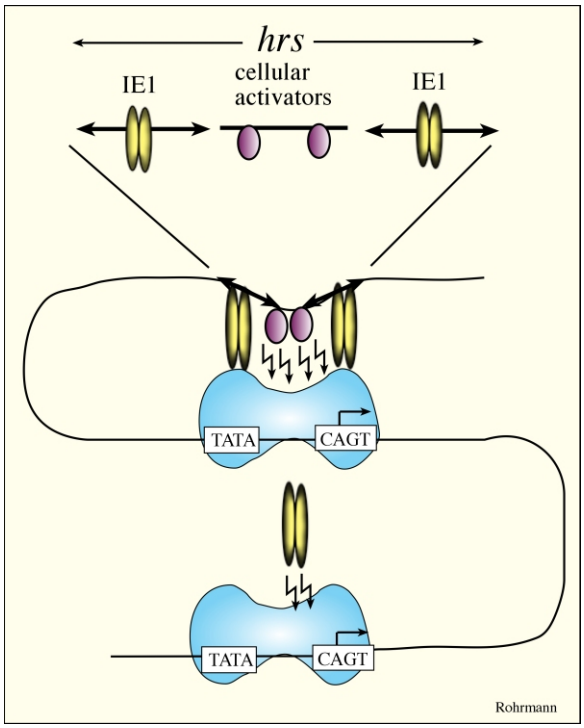

Fig. 4.3. Activation of baculovirus early gene transcription by *hrs* and IE1. This diagram shows two possible mechanism by which IE1 activates transcription. Transcription might be activated directly by IE1 as shown in the bottom of the diagram, or it can interact with both RNA polymerase II (in blue) and *hr* sequences thereby bringing *hr*-bound transcriptional activators in close proximity to RNA polymerase II as shown in the top of the figure.

upstream of the splice site [44]. AcMNPV IE1 is present as a homodimer but also can form aheterodimer with IE-0. Either IE1 or IE-0 can support the production of infectious virus; however, there are subtle differences in the timing of events and of BV and polyhedra production depending on which gene is being expressed suggesting that both IE-0 and IE-1 are required for wt levels of infection [37]. In contrast, in LdMNPV, only the spliced form is able to transactivate transient transcription and DNA replication [45].

Two additional transcriptional activators

AcMNPV appears to encode two additional factors that may be involved in activating transcription. It is likely that both these proteins form homodimers, but unlike IE1, specific DNA binding sites have not been identified suggesting that their activation may be based on their interaction with other proteins.

Ac151 (ie2/ie-n). Homologs of *ie2* are limited to Group I lepidopteran NPVs. IE2 contains a predicted RING finger domain indicating that it likely interacts with other proteins and in *B. mori* has been found to interact with itself [46]. IE2 was found to augment activation by IE1 [47-49] and was required for optimal origin specific plasmid DNA replication when transfected into Sf-21 cells, but had little effect in Tn-368 cells [50]. AcMNPV mutants deleted for *ie2* show a delay in infection in Sf-21. However, this delay was not observed in *T. ni* cells. In larvae of *T. ni* and *S. frugiperda*, the mutant viruses were significantly less infectious than wt which appeared to be due to the lack of virions in the occlusion bodies produced by the mutants. However, the intrahemocelic injection of mutant BV caused infections similar to wt [51]. IE-2 may also be involved in cell cycle regulation [52].

Ac153 (pe38). The other potential transcriptional activator in AcMNPV is *Ac153*. Homologs of Ac153 have an unusual distribution being found in all Group I NPV and four GV sequenced genomes. Ac153 was originally identified because of its early transcription profile and the presence of predicted zinc finger and leucine zipper motifs. It was named after a cDNA clone that hybridized to a PstI/EcoRI fragment [53]. Although it was not found to activate transient late transciption in one assay [54], evidence from OpMNPV indicated that it is a transactivator [55]. In addition, it appears to activate DNA replication in transient assays [35]. Deletion of AcMNPV *pe38* delays the infection and results in a 99% reduction in BV production, reduced levels of DNA synthesis, and a reduction in oral infectivity in larvae [56].

RNA polymerase II signals regulating early virus gene transcription

Upstream of baculovirus early genes are insect cell regulatory sequences including promoters that are recognized by the host cell RNA polymerase II and the associated transcriptional apparatus. The insect RNA polymerase II is able to initiate transcription from baculovirus promoters and even promoters that normally are transcribed by vertebrate cells such as the adenovirus major late promoter [57]. Two major sequences have been identified in baculovirus early genes including a TATA promoter motif and a transcriptional initiation consensus sequence, CAGT. Transient transcription of a minimal gp64 promoter was eliminated when either of these sequences was mutated suggesting that they both are necessary for transcription [58]. The CAGT sequence is apparently required for positioning the location where the mRNA sequence is started and can also act independently as an initiator. Initiators facilitate the assembly of the RNA polymerase II preinitiation complex and can promote transcription in the absence of a TATA sequence. This has also been demonstrated in baculoviruses for the *ie1* gene [59]. Many baculovirus early genes have TATA promoter sequences along with consensus CAGT (or CATT - see below) transcriptional initiation sequences located about 30 bp downstream. These consensus sequences are two of the most prevalent motifs found in regulatory regions of genes from *Drosophila* [60]. Several sequences have been identified both upstream and downstream of the promoter region that elevate early gene transcription; however the mechanism underlying their function has not been characterized [61]

Genome-wide analysis of baculovirus promoters

A survey of predicted baculovirus promoter sequences was conducted on 26 baculovirus genomes [62]. In this investigation, sequence patterns were characterized for the 300 bp of DNA upstream of the ATG initiation codons of baculovirus orfs. The significance of the prevalence of these predicted promoter sequences was determined by comparing their frequency in the 300-bp downstream of the ATG. There was a strong correlation for TATA sequences upstream of an ORF. Particularly significant were sequences such as TATAAGG that contain both TATA and late promoter sequences (ATAAG) in combination. These were about 7 times as frequent in the upstream location. Furthermore, about 70% of the TATA sequences present in the genome are clustered in the 100-bp upstream of the ATG. TATA sequences combined with mRNA start site

consensus sequences CAGT or CATT separated by ~30-bp were 3 times as frequent in the upstream region. Similarly, putative initiator sequences [63] in combination with a TCGT motif ~30-bp downstream were almost 5 times more prevalent upstream of the ATG. Some other sequences such as GTAG, AGTC and TAGG were about 20 times more frequent in the promoter (upstream) region. They were often present in combination with early or late promoter elements suggesting that they are involved in optimizing transcription from these sequences.

How are baculovirus early genes activated?

IE1 can activate transcription and the level of activation is increased many fold in the presence of *hr* enhancer sequences. In addition, *hr* sequences appear to be capable of binding to cellular factors that activate transcription. Since IE1 can activate transcription in the absence of *hrs*, it likely has the ability to interact with the RNA polymerase II-containing transcription complex and facilitate either the recruitment of other factors, or to elevate the levels of transcription itself. Since *hrs* appear to bind both cellular transcription factors and IE1, IE1 could recruit the *hr* bound factors to the RNA polymerase II complex (Fig. 4.3). In this manner it would appear to be able to influence the rate at which RNA polymerase is recruited to or stabilized at a specific promoter and its subsequent ability to efficiently transcribe RNA.

Shutoff of early transcription

A feature of most baculovirus early genes is that they appear to be transiently expressed and are shut off at late times postinfection. The mechanism for this shutoff is not understood. Some early genes avoid being shut off early by having late in addition to early promoter elements. These genes can be continuously expressed through both early and late times postinfection. In addition, some genes such as *ie1* appear to be transcribed throughout infection by an undetermined mechanism [26].

A mechanism for the regulation of RNA polymerase II entails the addition or removal of phosphates to its carboxyl terminal domain (CTD) and CTD phosphatases are involved in this process. Ac98 has homologs in all baculovirus genomes and although its function has not been determined it is required for viral viability [64], and may stimulate both early and late gene expression in transient assays [65]. When put through the HHpred program [66] that compares predicted proteins to known structures (see Chapter 6), Ac98 showed relatedness to a set of enzymes including CTD phosphatases with a probability of almost 100%. Dephosphorylation of the CTD can negatively regulate RNA polymerase II by inhibiting RNA elongation [67, 68]. Although Ac98 may stimulate early transcription in a transient assay, this needs to be investigated in the context of viral infections as Ac98 itself could be regulated to selectively stimulate baculovirus early genes.

Caveats and qualifications

Despite the complexity of the structure of baculovirus virions and the presence of nonstructural proteins that are likely packaged with budded and occluded virions, it is possible that none of these proteins are required for the initiation of infection once the DNA is uncoated in the nucleus. Consequently, although many proteins have been reported to be associated with baculovirus virions, none have been shown to be required for the initiation of gene expression or DNA replication. Additional evidence for independence from virion proteins is the observation that purified viral DNA devoid of proteins is capable of initiating baculovirus infection when transfected into susceptible cells [69] [70] [71] [72]. Therefore, no viral proteins are required to initiate a baculovirus infection; however this observation does not rule out the possibility that they facilitate the efficiency of the infection. In fact, IE1 was found to be associated with BV but not ODV of OpMNPV [73].

Another caveat regarding the molecular events involved in viral transcription and replication is that almost all this information is derived from AcMNPV, and to a lesser extent, the closely related BmNPV. Whereas much of this information is likely applicable to most baculoviruses, there are major differences, not only in gene content, but also in gene sequence homology between AcMNPV and many other baculoviruses. In addition, there are major differences in the number and distribution of *hr* enhancer sequences. All these factors likely have major, but currently unknown, influences on these events in other baculoviruses.

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Chapter 5
DNA replication and genome processing

Cells encode all of the genes necessary for the replication of their own DNA. Viruses that infect these cells can either exploit this apparatus for their DNA replication, or they can independently encode some or all the genes involved in this process. However, it is likely that most viruses that replicate in nuclei use a combination of host and virally encoded proteins. Determining the viral factors involved in DNA replication provides fundamental information on how viral DNA synthesis is accomplished. In addition, because so much is known about the proteins involved in DNA replication in other systems, the identification of proteins that are encoded by the virus can lead by inference to proteins that are likely contributed by the host cell.

Some questions are fundamental to understanding viral DNA replication. These include: i) the identification of sequences, called origins of replication, that specify where DNA replication begins; ii) the identification of gene products involved in DNA replication; iii) understanding the role of these proteins in DNA replication; iv) understanding how the DNA is replicated; and v) understanding how the DNA is incorporated into nucleocapsids. Rapid advances in the first three of these areas were made for baculoviruses by the application of approaches developed for similar studies in other viruses. However, these methods often employ transient assays that involve the transfection of combinations of cloned viral DNA into insect cells, and although they have yielded significant information, they do not result in the production of mature, functional genomes. Below is a summary of the current information in this area.

Identification of origins of viral DNA replication

When a DNA virus that replicates in nuclei infects a cell, it must distinguish its DNA from that of the host in order to facilitate specific replication of viral DNA, rather than that of the host. One method of distinguishing viral DNA from host DNA is the presence of unique viral DNA sequences that specify sites for the initiation of DNA synthesis. These sequences are called origins of replication. By directing the synthesis of a protein that recognizes a viral replication origin, a virus can specify DNA sequences that will be replicated.

Two approaches have been employed to identify viral origins of DNA replication. In one, plasmids that contain viral DNA are transfected into virus infected cells. To distinguish newly replicated DNA from input DNA, a restriction enzyme (DpnI) is used because it cuts at a site that is methylated in the input plasmid DNA (derived from bacteria), but not at that site in DNA that has replicated in insect cells because it is not methylated. Therefore, if a plasmid is replicated in insect cells, it is not methylated at its DpnI sites and therefore is resistant to DpnI and is not digested by the enzyme. The other approach is to pass the virus at high multiplicity of infection (moi). This results in the enrichment of defective genomes with major deletions that replicate rapidly due to their small size. They rely on the presence of complete genomes with a full complement of replication genes to support their replication. The defective genomes are often very small and are enriched for replication origins because these sequences are preferentially amplified and can, in some instances, evolve to become the most prevalent sequences produced after extended high moi viral passage. Both these approaches identified homologous regions (*hrs*, see Chapter 4) as causing the infection-dependent replication of plasmids [1, 2]. However, using the same two techniques, non-*hr* sequences that acted as origins of replication were also identified [3, 4]. Subsequently, early promoters were also identified as having origin activity [5]. Using another type of assay based on PCR of DNA extracted from infected cells, both a non- *hr* origin and an early promoter were shown to have origin activity [6]. Consequently, although *hr* and non- *hr* origin sequences have been examined in some detail, it is still not clear which, if any, of these sequences is the preferred site of replication initiation in vivo.

It is possible that DNA replication may initiate at any sequences that become unwound thereby providing an entry point for the replication

complex. The baculovirus transcriptional transactivator IE-1 binds to *hr* sequences and may be involved in facilitating entry at these sites. Likewise, RNA polymerase II would cause unwinding at early promoter sequences. Non-*hr* origins may provide sequences of less specificity that are involved in DNA unwinding. However, understanding the role of these sequences is complicated by a lack of understanding of the structure of baculovirus replication intermediates. All available information suggests that baculovirus DNA is replicated either by a rolling circle mechanism [7, 8] or one that includes extensive recombination or a combination of both these mechanisms [9]. If recombination-dependent replication and rolling circle mechanisms are major contributors to baculovirus genome amplification, then origins of replication ultimately may play a minor role in this process. Once genome replication is initiated, subsequent amplification might be independent of specific sequences.

Genes required for DNA synthesis

The genes involved in baculovirus DNA synthesis were originally identified by a combination of the characterization of temperature sensitive mutants [10] and by transient replication assays [11, 12]. Transient replication assays involve the transfection of regions of the viral genome along with a plasmid containing a replication origin and the use of the DpnI digestion assay described above. Starting with enough of the genome to give positive replication, subsequent experiments involve the elimination of sequences until a minimal set of genes is identified. Since the original experiments were conducted on AcMNPV before the complete genome sequence was published, the final set of plasmids was sequenced which allowed identification of the essential genes. Six genes were identified that are essential for transient DNA replication and several others were found to be stimulatory. The essential genes include *dnapol*, *helicase (p143)*, *lef-1 (late expression factor-1)*, *lef-2*, *lef-3* and *ie1* (Table 5.1). In addition to *dnapol* and *helicase*, these genes have the following functions: LEF-1, primase; LEF-2, primase accessory factor; LEF-3, single stranded DNA binding protein (SSB); and IE-1, unknown but it may bind to origins of replication. Homologs of the first four have been identified in all sequenced baculovirus genomes [13]. The lack of homologs of *lef-3* and *ie1* does not necessarily imply their absence in evolutionary distant genomes. It could reflect the incorporation of so many changes that the homology of the primary sequences can no longer be identified. This set of genes is similar to those identified as being required for herpes simplex 1 transient DNA replication [14] (Table 5.1). In both these viruses it is likely that host enzymes also participate in this process. The similarity of the complement of herpes virus and baculovirus replication and other genes has led to the suggestion that these virus are members of a lineage distinct from other large DNA viruses of eukaryotes [15]. Table 5.1. Viral genes essential for DNA replication

Gene	Baculovirus	Herpesvirus
DNA pol	+	+ (UL30)
Ssb	+ (LEF-3)	+ (ICP8)
primase	+ (LEF1)	+ (UL52)
Primase accessory factor	+ (LEF2)	+ (UL8)
helicase	+ (p143)	+ (UL5)
Origin binding protein	+ IE1 (?)	+ (UL9)
Processivity factor	-	UL42

Initiation of DNA replication in well-characterized systems such as *E. coli* is an orderly process that begins with an origin binding protein interacting with an origin of replication and unwinding this region, thereby allowing access by a helicase that induces more extensive unwinding. This causes recruitment of the primase, followed by primer synthesis that then attracts the DNA polymerase. Many of the factors involved in baculovirus DNA replication are similar to those found in other organisms and are described below starting with IE1 that has properties somewhat similar to an origin binding protein (also see Fig. 5.1).

IE1 (Ac147). IE1 has been identified in all NPV genomes sequenced from the Lepidoptera and also appears to be present in GV genomes, but the homology is very low. Homologs have not been identified in the hymenopteran and dipteran virus genomes that have been sequenced [13]. As described in Chapter 4, IE1 is a transcriptional activator that binds to *hr* sequences. The identification of *hrs* as putative replication origins and of IE1 as an *hr* binding protein would suggest that IE1 is an origin binding protein and might serve as a marker for enucleating the assembly of a replication complex. This would allow viral DNA to be distinguished from host DNA and would lead to the specific replication of viral

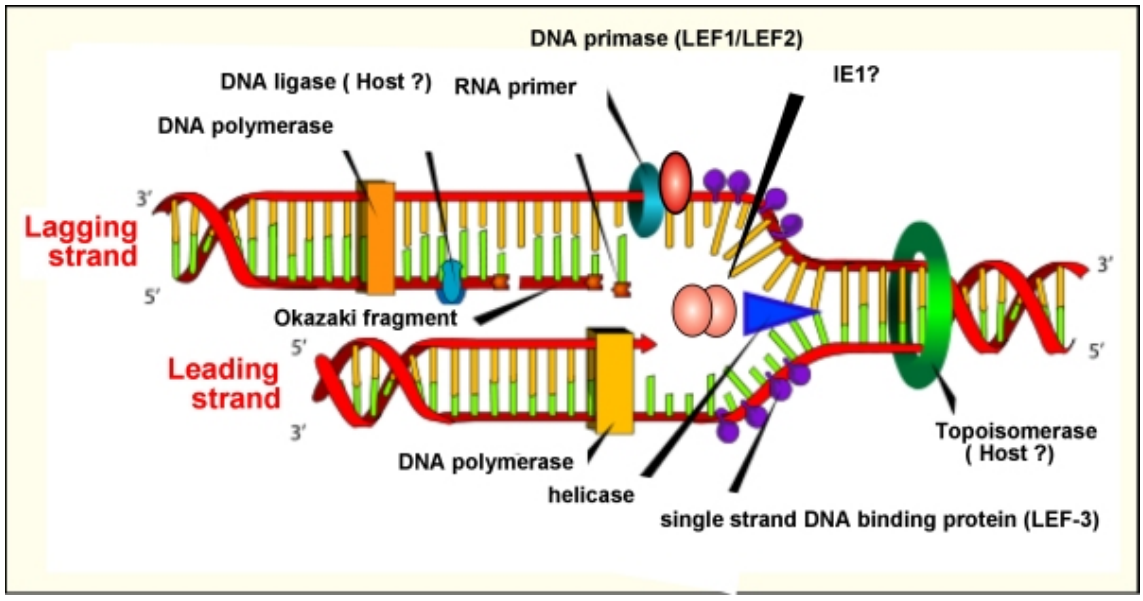

Fig. 5.1 AcMNPV DNA replication. This diagram shows a hypothetical relationship of the baculovirus replication factors along with several factors, DNA ligase and topoisomerase that are likely contributed by the host. IE1 is also shown, although its role is unknown. Diagram courtesy of and modified from a figure by Mariana Ruiz.

sequences. However, the identification of non-*hr* sequences that also act as replication origins and to which IE1 does not appear to specifically bind, suggests that IE1 might not be an origin binding protein in all cases. Because IE1 acts as a transcriptional transactivator of baculovirus early genes, and since all the viral genes involved in DNA replication are early genes, IE1 could act as a transactivator of all these genes. Without transactivation by IE1, the replication genes might not be synthesized to a high enough concentration for transient DNA replication to be detected.

LEF-3 (Ac67), a single-stranded DNA binding protein (SSB). A feature of DNA replication systems is a dependence on the presence of SSBs. SSBs bind tightly to ssDNA and prevent the formation of secondary structure. They also prevent the renaturation of ssDNA and may inhibit non-specific interactions of ssDNA with other proteins. They bind in a cooperative manner and can interact with replication proteins. AcMNPV LEF-3 was identified as being required for DNA replication and was subsequently characterized as an SSB [16]. It is a 385 aa protein with a molecular weight of about 44.5 kDa. LEF-3 homologs are present in all lepidopteran baculovirus genomes, but have not been reported in baculoviruses from Hymenoptera or Diptera [13]. A LEF-3 homolog may very likely be present in these viruses, but its primary amino acid sequence may have evolved to such an extent that it is not readily identified. For example, the putative XecnGV LEF-3 and AcMNPV LEF-3 show only 14% aa sequence identity.

In addition to being an SSB, LEF-3 has a number of other roles. LEF-3 interacts with alkaline nuclease and may regulate the function of this enzyme [17, 18]; it is capable of both unwinding and annealing DNA depending on its concentration or redox state [19, 20]; and it can facilitate the production of recombination intermediates via strand exchange between donor and recipient molecules in vitro [21]. LEF-3 binds to other LEF-3 molecules which may facilitate its function. LEF-3 also interacts with helicase and is required for its transport into nuclei [22]. The nuclear localization signal was localized to about 5 amino acids and was also found to facilitate nuclear transport in mammalian

cells [23]. LEFF-3 is also required for DNA replication independent of this role [24]. This feature was localized to the N-terminal 125 amino acids [25]. Since SSBs are often required for DNA replication, it may have two roles in this process--to mediate helicase transport into the nucleus and as an SSB. When DNA replication is initiated, one can envision the unwinding of DNA at a specific location. LEF-3 may then bind to the ssDNA and could transport helicase to this site and bring it into close proximity to this ssDNA (see below).

Helicase (p143) (Ac95). DNA helicases are critical enzymes involved in DNA replication. They are motor proteins that move directionally and unwind DNA by disrupting the hydrogen bonds between bases in the double helix. This exposes the bases so that complementary DNA strands can be synthesized. Energy provided by the cleavage of ATP by an ATPase domain in helicase is required for strand separation.

A helicase homologue was identified in an AcMNPV ts mutant that was defective in DNA replication [10]. Helicase was subsequently found to be required for DNA replication in transient assays and was shown to have the biochemical properties of a DNA helicase with both ATPase and helicase activities, and the capacity to bind ss and dsDNA [26]. As described above, helicase interacts with LEF-3 and this could facilitate the interaction of helicase with ssDNA.

A DNA primase, LEF-1 (Ac14) and a primase-associated factor, LEF-2 (Ac6). Once a segment of DNA is unwound and stabilized by its interaction with SSBs, DNA replication can be initiated. However, DNA polymerases are unable to independently initiate the synthesis of a new strand of DNA; they can only elongate from an -OH group on an existing strand. Therefore, a short sequence of oligonucleotides that serves as a primer must be synthesized first. Primers are generated by a category of RNA polymerases called primases. This process results in hybrid molecules containing small regions of RNA linked to DNA. Eventually, the RNA primer is removed and replaced by DNA.

In AcMNPV, LEF-1 and LEF-2 were originally identified as factors required for DNA replication in the transient assay system. Subsequently it was found that LEF-1 interacts with LEF-2 [27]. It was originally suspected that LEF-1 was a DNA primase because it contained a conserved primase domain (WVVDAD). When this domain was altered to WVVQAD, transient DNA replication activity was eliminated [27]. Purified LEF-1 was found to have primase activity and products of several hundred nucleotides or more were synthesized when M13 ssDNA was employed as a template. Elution profiles of LEF-1 and LEF-2 from ssDNA cellulose and DEAE resin suggested that LEF-2 may bind to both DNA and LEF-1. Although it is essential for transient DNA replication, the exact role of LEF-2 has not been clearly defined [28].

Based on limited amounts of DNA synthesis by a *lef-2* bacmid knockout in transfected cells, it was suggested that Lef-2 is not required for the initiation of DNA replication. This was in contrast to a bacmid with the helicase gene deleted that showed no synthesis [29]. However, the data in this report could be interpreted in a different manner. The transfected DNA is likely nicked and therefore the ends of the nicks could act as primers resulting in limited amounts of leading strand DNA synthesis in the absence of an active primase complex. A control with the helicase deleted showed no DNA synthesis indicating that it is required for all DNA synthesis.

Phylogenetic analysis suggests that LEF-1 and LEF-2 are members of a primase lineage common to archaea and eukarya that is distinct from the primase lineage of a number of other large eukaryotic DNA viruses [30].

Baculovirus primers. In contrast to leading strand synthesis, Lagging strand synthesis of double-stranded DNA is not continuous and results in the synthesis of non contiguous oligomers called Okazaki fragments that fill in the single stranded region generated as the leading strand is synthesized (Fig. 5.1). These relatively short oligomers are transient because they become ligated to the lagging strand. When short-lived DNA intermediates were characterized in *S. frugiperda* cells, fragments of 80-200-bp were observed. These are similar in size to the Okazaki fragments observed in other eukaryotic cells. However, when these intermediates were characterized in AcMNPV infected cells, they were much larger (0.2 – 5 kb) with an average of 1-2 kb. This is similar in size to what has been observed in prokaryotes, bacteriophage, and herpes virus and may reflect a lower frequency of priming than the host cell.

This would result in longer fragments being synthesized to span the distance between the primed regions [31].

DNA polymerase (Ac65). As described above, DNA polymerases extend DNA from the 3' -OH of an RNA primer in a 5' to 3' direction. This results in the synthesis of a leading strand that can continue indefinitely. However, synthesis of the second or lagging DNA strand must occur in the opposite direction of the leading strand in order to progress in a 5' to 3' direction. As the leading strand is synthesized, additional DNA is exposed on the complementary strand. Consequently, whereas leading strand synthesis can be continuous, lagging strand synthesis occurs in segments called Okazaki fragments.

DNA polymerases often have associated exonuclease activities. A 3' to 5' exonuclease activity allows the removal of newly synthesized DNA in the reverse direction of synthesis. This activity is associated with proof reading and allows the polymerase to correct mistakes as it is synthesizing DNA. This may cause DNA polymerase to repeatedly shuttle between polymerizing and editing modes [32]. The other activity involves a 5' to 3' exonuclease and is used for the removal of RNA primers. This would be particularly important in lagging strand synthesis because the extended strands would invariably collide with the RNA primer of the upstream Okazaki fragment as they synthesize DNA in the direction opposite of the replication fork. Some DNA polymerases such as DNA pol I of *E. coli* have both 3' to 5' and 5' to 3' exonuclease activities so they can proofread and repair newly synthesized DNA and also remove RNA primers. However, many DNA polymerases lack the 3' to 5' activity.

All DNA viruses that infect animal cells appear to encode their own DNA polymerases, except for some with small genomes (polyoma, papilloma and parvoviruses). It has been suggested that this benefits the virus because there may be insufficient levels of the cellular DNA polymerase in infected cells since they are not dividing, and therefore, are not in S phase [33]. In fact it has been shown that the mRNA and protein levels of several DNA polymerases increase by three fold or more during S phase [34]. However, the benefits of viruses encoding their own polymerases could also be due to the isolation of viral DNA replication to specific foci within nuclei, or simply that it is more efficient to have the DNA polymerase under regulatory control of the virus so that its expression can be coordinated with the infection. A baculovirus specific DNA polymerase was originally characterized from *Bombyx mori* infected with BmNPV [35]. Subsequently a gene with homology to DNA polymerase was identified in the AcMNPV genome using hybridization of primers to conserved DNA polymerase sequences [36]. This gene is most closely related to members of the DNA polymerase B family. Enzymes in this family synthesize both leading and lagging strands of DNA and have a high degree of fidelity that is associated with a strong 3' to 5' exonuclease activity. The enzymes from BmNPV and AcMNPV were shown to have a 3' to 5' exonuclease activity [35, 37, 38], and therefore likely have the capacity to proofread newly synthesized DNA and remove DNA sequences if defects such as mismatching are detected. In contrast, a 5' to 3' exonuclease activity was not observed [38]. Since this activity is associated with removal of the primer used for the initiation of DNA synthesis, it is not clear how primers are removed during baculovirus DNA replication (see below).

An unexpected observation described in one report employing transient assays for the identification of baculovirus replication genes suggested that DNA polymerase was not essential, since significant levels of DNA replication (12%) were observed in the absence of *dnapol* [12]. This led to the suggestion that it might be stimulatory rather than essential and that the host DNA polymerase may be involved in initiating baculoviral DNA replication. Furthermore, it was observed that in transient replication assays the AcMNPV DNA polymerase could be substituted with the ortholog from OpMNPV [39] or even from an ascovirus [40]. This further suggested that the DNA polymerase may be interchangeable in the context of the other AcMNPV replication proteins. However, when the DNA pol gene was deleted from a bacmid containing the AcMNPV genome, no viral DNA replication was observed [41]. This suggested that the DpnI resistant DNA generated in the absence of DNA polymerase in the transient assays could be due to repair of the origin containing plasmid caused by the other baculovirus genes and a host cell DNA polymerase.

Additional genes that influence DNA replication

A variety of other genes have been identified that influence DNA replication, but their role in this process has not been characterized. These include the following:

DBP (Ac25), A second SSB. AcMNPV encodes two proteins that possess properties typical of SSBs, LEF-3, and a protein referred to as DNA-binding protein (DBP). Homologs of dbp are found in all sequenced baculovirus genomes except that of the dipteran (CuniNPV) and in some instances multiple copies of the dbp gene are present. It has properties similar to LEF-3 in that it interacts with itself and is capable of both unwinding and annealing DNA [42]. DBP was also able to compete with LEF-3 for binding sites on ssDNA templates and protected ssDNA against hydrolysis by a baculovirus alkaline nuclease (AN)/LEF-3 complex. It is an essential gene, as bacmids lacking Ac25 were non infectious and appeared to produce defective nucleocapsids; however, unlike LEF-3 it is not required for transient DNA replication. Although not a virion structural protein, fractionation studies indicated that DBP is tightly associated with subnuclear structures suggesting that it is a component of the virogenic stroma [42]. It was found to localize to the virogenic stroma by immuno-electron microscopy and when *dbp* was deleted from an AcMNPV bacmid, cells transfected with this construct appeared to lack a virogenic stroma and failed to produce normal-appearing nucleocapsids. This suggested that *dbp* is required for the production of nucleocapsids and the virogenic stroma [43]. In addition, although viral DNA synthesis occurred in cells transfected with the *dbp* knockout, the levels were less than that of the control virus indicating that DBP may be required for normal levels of DNA synthesis, or for stability of nascent viral DNA. Analysis of the viral DNA replicated by the *dbp* knockout by pulsed-field gel electrophoresis resulted in DNA of apparent high molecular weight that is retained in the wells of the gel and fragments that are shorter than the full-size viral genome [43]. The DNA fragments might represent degradation products of viral genomes or replicative intermediates. Two different activities of DBP might be responsible for these observations. First, DBP may prevent the enzymatic degradation of viral genomes. It has been shown that DBP inhibits hydrolysis of DNA by the proofreading activity of phage T4 DNA polymerase [44] or by the nuclease activity of the AN/L3 complex [42]. Therefore, DBP may protect mature viral genomes against nucleases and stabilize them at stages that precede packaging into virions. On the other hand, the unwinding and renaturation activity of DBP may be required for processing of replicative intermediates by annealing and strand invasion reactions involved in DNA recombination that may be essential for the complete replication and processing of baculovirus genomes [9, 45, 46].

LEF-11 (Ac37). Homologs of LEF-11 are present in all baculovirus genomes except the dipteran CuniNPV. It was found to stimulate late gene transcription, but was not required for transient DNA replication [12]. However, an AcMNPV bacmid deleted for lef-11, was unable to synthesize DNA or carry out late gene transcription when transfected into Sf-9 cells [47]. No homology to known proteins associated with DNA replication was identified in the Lef-11 sequence.

Ac139 (ME53). Homologs of Ac139 are present in the genomes of all lepidopteran baculoviruses. AcMNPV bacmids deleted for this gene are not viable, fail to replicate their DNA, and do not produce nucleocapsids. However, cells transfected with DNA from the deletion mutant showed early stages of cytopathogenic effects including nuclear enlargement and the formation of granular material in the nucleus [48]. No homology to known proteins associated with DNA replication was identified in the Ac139 sequence.

Lef-7 (Ac125). Lef-7 is stimulatory for transient DNA replication [12, 49]. When deleted, infection was unaffected in Tn368 cells, but in Sf21 and *S. exigua* cells DNA replication was 10% of wt [50]. When deleted from BmNPV, a reduction in DNA synthesis was also observed [51]. Homologs are present in all Group I, three group II NPV, and two of ten sequenced GV genomes.

Proliferating cell nuclear antigen (PCNA) (Ac49). Ac 49 has homology to proliferating cell nuclear antigen (PCNA). PCNA homologs have been found in the genomes of four Group I (not BmNPV) and two Group II lepidopteran NPVs and appear to have been derived from two different lineages (see Chapter 12). Although eukaryotic PCNA lacks an enzymatic function, it plays a role in DNA synthesis, DNA repair, and cell cycle progression. It functions as a sliding circular clamp that mediates protein interactions

with DNA and is required for the coordinated synthesis of both leading and lagging strands at the replication fork during DNA replication [52]. In AcMNPV it is not an essential gene [53, 54] and did not appear to elevate DNA replication in transient replication assays [11]. However, it did appear to be involved in the timing of late gene expression [55].

Baculovirus DNA replication genes: What's missing?

Topoisomerase. Based on the general model of DNA replication, baculoviruses encode most of the genes involved in this process (Fig. 5.1). However, there are two major factors that have not been identified. These include a topoisomerase and a DNA ligase. As helicases advance and unwind DNA they cause an increase in the number of twists or topoisomers that accumulate in the DNA in advance of the enzyme. This can cause a barrier to the advance of the helicase. This problem is alleviated by topoisomerases that break and rejoin DNA strands thereby allowing the DNA helix to unwind and relieving the barrier to helicase progression. Integrase homologs from other viruses show topoisomerase activity [56]. Therefore, it was thought that VLF-1, a homolog of integrase encoded by all baculovirus genomes, may be involved in this process; however purified VLF-1 showed no topoisomerase activity [57]. It is possible that either VLF-1 might carry out this function as a complex with another, unidentified protein, or that a host topoisomerase is involved in this process.

Dealing with Okazaki fragments: two DNA ligases and a second helicase. Another enzyme that has not been identified in most NPV genomes is a DNA ligase. This enzyme is necessary for the ligation of Okazaki fragments to one another. For viruses lacking a DNA ligase homolog, it is assumed that a host enzyme supplies this function. However, ligase homologs are present in all granulovirus genomes that have been sequenced and in two NPV genomes (LdMNPV and MacoNPV-B). The DNA ligase of LdMNPV has been shown to have enzymatic activity and is able to form a covalent link with [α32P]ATP and to ligate double-stranded synthetic DNA substrates containing a single nick suggesting that it would be capable of ligating Okazaki fragments. It was not required or stimulatory for DNA replication in transient replication assays, although these assays may not be dependent upon ligase activity [58]. A striking feature of the baculovirus ligase homologs is that they are always (except MacoNPV-B) accompanied with a helicase homolog that is not found in any of the genomes lacking ligase. This helicase is related to the PIF1 family [58] (note: this is not a per os infectivity factor). Members of this family have a preference for RNA-DNA hybrids and could be involved in the maturation of Okazaki fragments [59]. This may involve displacement of the RNA primer producing an RNA flap that would then be cleaved by a flap endonuclease (FEN) [60] or digested by a 5' to 3' exonuclease. DNA polymerase would then fill in the gap by extending the Okazaki fragment and the ligase could join the fragments. Another enzyme that may be a candidate for a DNA ligase in some viruses is Ac105 (also called HE65). Computer predictions indicate that it contains a domain homologous to eukaryotic DNA ligases. Homologs of Ac105 are present in some Group I and II NPVs and two GVs. Therefore, although there are at least two candidate ligases, it is not clear what role they play in baculovirus DNA replication. Ac105 is probably non essential as insertion/deletion of this gene in BmNPV (Bm89) had no apparent effect on infectivity [61].

Rationale for the composition of baculovirus replication genes

As described above, baculoviruses and herpes viruses encode many, but not all, genes that are likely to be involved in DNA replication. To understand the evolution of these systems, it is important to determine the theoretical rationale for the genes that these viruses encode. A main reason for encoding replication genes rather than using host genes, would be to separate the viral genome replication from dependence on the host cell. Since most host genes are likely shut down upon infection [62], encoding a replication system independent of host cell control may be of compelling importance. However, this still does not explain the pattern of replication genes that are virally encoded. As shown in Table 5.1, these two DNA viruses encode a DNA polymerase, an SSB, and a primase. The presence of the SSB may be due to a requirement for its abundant expression, because of all the replication proteins, it may have to be present in the highest concentration (Fig. 5.1). In addition, the presence of virally encoded DNA polymerase/primase may also be due to the need

for optimal concentrations higher than the host cell can provide--especially if the host is stalled at the G2/M stage by the viral infection (see Chapter 7). In particular, each nucleotide polymerized would have to be manipulated by the polymerase. Likewise, each nucleotide of the DS DNA would have to be separated by the DNA helicase. In contrast, the lack of the necessity for a viral encoded ligase could be due to it being required only rarely, especially if long Okazaki fragments are produced as may be the case (see above) [31]. Under these circumstances, the low levels of ligase present in the host cell may be sufficient. In addition, if the viral DNA is nicked and not covalently closed during replication, a topoisomerase activity may not be required to relieve torsional stress during replication of the virus genome. However, it would be necessary for the production of the final covalently closed supercoiled DNA that is packaged into virions. Whether this requires only low levels of a host enzyme remains to be determined.

Location of baculovirus DNA replication; development of the virogenic stroma

As described in Chapter 3, the virogenic stroma is the site of viral genome replication and nucleocapsid assembly. Several investigations have examined the viral gene products involved in the formation of this structure. IE-1 appeared to localize to specific foci before DBP and LEF-3. After DNA replication begins, the foci enlarge and occupy over half the nucleus and DBP, IE-1, and LEF-3 along with newly replicated DNA co-localize to this region. When DNA replication was inhibited with aphidicolin, foci containing all three proteins were present at early times post infection, but were not as uniform as in the absence of the drug. The number of IE-1 foci appears to be restricted to about 15, suggesting that there are a limited number of preexisting sites where DNA replication could be initiated. It was suggested that these might be equivalent to nuclear domain 10 (ND10) sites found in mammalian cells [63]. ND10 are sites of concentrations of proteins involved in a variety of cellular processes and these sites are often associated with virus assembly [64, 65]. Similar data on the localization of replication proteins has been described by others, and it was suggested that IE2 may also be associated with these sites [66]. Subsequently it was found that *hr* sequences were sufficient to cause IE1 to form foci [67] and that the presence of the viral DNA helicase was necessary for LEF-3 to localize to these structures [68]. This is consistent with the requirement of LEF-3 for the transport of helicase into nuclei described above [22]. In addition, it was suggested that the replication factors DNA helicase, LEF-3, IE1 along with *hr* sequences are all that is required to produce foci capable of recruiting other replication factors [68]. Studies on the relationship of the IE1 foci with virion structural proteins indicated that ODV-E25 (Ac94) and vp91 (Ac83) localize to the periphery of these structures, whereas vp39 was found within the IE1-associated foci [69]. It has also been suggested that IE1 interacts with another structural protein BV-ODV-E26 (Ac16) and serve to recruit it to replication sites [70]. Using fluorescent-tagged histone H4, the effect of baculovirus infection of this histone was examined. It was found to relocate to the margins of infected nuclei and appeared to be excluded from the viral replication compartment. This marginalization of histone H4 could also be induced by ie1, lef3, p143-helicase and an hr [71].

Additional baculovirus genes: hints of DNA repair

Although, as described above, the 3' to 5' exonuclease activity of the baculovirus DNA polymerase suggests that it has the ability to proofread DNA as it is synthesized, there do not appear to be other DNA repair systems common to all baculoviruses. However, there are enzymes produced by a limited set of baculoviruses that suggest that some viruses may encode additional pathways for repairing their DNA. This suggests that viruses lacking these pathways may be able to co-opt the homologous proteins from the host cell.

Photolyase. A common threat to occluded baculoviruses in the environment is their inactivation by UV light [72]. UV light causes crosslinking of adjacent pyrimidine residues. These crosslinked dimers can cause the DNA to bend and this may inhibit the ability of the DNA replication complex to copy beyond the damaged site or cause the incorporation of incorrect nucleotides. This may result in lethal mutations or the inhibition of the interaction of proteins involved in gene regulation. Photolyases are enzymes that bind to the site of such mutations

and, after being activated by light, can catalyze the separation of the mutant dimer, thereby correcting the mutation. Homologs of photolyase genes have been found in the genomes of Group II baculovirus that are members of a lineage that infects insects of the subfamily Plusiinae of the family Noctuidae [73] [74] [75]. *Chrysodeixis chalcites* encodes two photolyase genes that are predicted to encode proteins with 45% amino acid sequence identity. When tested, one copy showed photoreactivating activity, whereas the other copy did not [76]. Transfection of egfp fusions of photolyase genes into *T. ni* cells, resulted in fluorescence localized to chromosomes and spindles and other structures associated with mitosis. Baculovirus infection of the transfected cells caused fluorescence to localize to the virogenic stroma [77]. The incorporation of an algal virus photolyase gene as a means to cause resistance to UV inactivation of AcMNPV has been described. However, although BV survival was increased after exposure to UV light, occluded virion survival was not [78].

dUTPase. Deoxyuridine triphosphate (dUTP) can be mutagenic if incorporated into DNA. The enzyme dUTPase dephosphorylates dUTP to dUMP, which is a substrate for thymidine biosynthesis. Homologs of dUTPase are present in 10 NPV and one GV genome [79]. Baculoviruses may have incorporated this gene to either supplement or substitute for the host gene. The viruses that encode a *dUTPase* homolog also normally encode both subunits of ribonucleotide reductase (RR). The presence of RR may have selected for the incorporation of *dutpase* to mitigate the production of the dUTP mutagen by ribonucleotide reductase.

Viral three-prime repair exonuclease (v-trex). A gene with homology to 3' to 5' exonucleases from other systems has been identified in two baculovirus genomes. The enzyme from both AgMNPV and CfMNPV demonstrated 3' to 5' exonucleolytic activity [80, 81]. These enzymes would appear to carry out proof reading functions similar to those employed by the DNA polymerase. However, it has been suggested that trex proteins may associate with DNA polymerase and increase the fidelity of DNA replication under conditions that cause error prone nucleotide polymerization [32].

Ac79, a member of the UvrC endonuclease superfamily? It has been suggested that Ac79 is a member of the UvrC superfamily of endonucleases that are involved in DNA repair [82]. Homologs of Ac79 are present in all group I and about half group II lepidopteran NPV and also in a few GV genomes. It has homology to orfs found in other insect viruses and a variety of bacteria. It is predicted to be homologous to an endonuclease in a number of these organisms. It is likely essential because interrupted mutants of this gene in BmNPV (Bm65) could not be isolated [61]. Ac79 was found to be associated with AcMNPV ODV [83].

PARP. A homolog of poly (ADP-ribose) polymerase (PARP) is found in one baculovirus genome (AgMNPV – Ag31) [84] [85]. PARP is an enzyme found in nuclei that is activated by DNA strand breaks and uses NAD+ as a substrate to synthesize polymers of ADP-ribose on acceptor proteins that are involved in the repair of single strand breaks in DNA by activating and recruiting DNA repair enzymes. It is also involved in telomere elongation, chromatin structure, and the transcription of a variety of genes involved in immunity, stress resistance, hormone responses, and the possible silencing of retroelements [86].

PARG. Poly (ADP-ribose) glycohydrolase (PARG) is an enzyme that reverses the products produced by PARP [87]. PARG is the primary enzyme responsible for the catabolism of poly(ADP-ribose) in vivo. Therefore, whereas PARP stimulates a variety of processes, PARG reverses the products of PARP. Homologs appear to be present in all sequenced lepidopteran group II NPV genomes.

Genes involved in nucleotide biosynthesis

Most baculoviruses do not encode genes involved in nucleotide biosynthesis. However many Group II and several GVs encode both subunits of ribonucleotide reductase. In addition, these same viruses normally also encode dUTPase (see above).

Ribonucleotide reductase. Ribonucleotide reductase is a heterodimer composed of large and small subunits (RR1 and RR2, respectively). It is involved in the catalysis of ribonucleotides to deoxyribonucleotides as a pathway for providing nucleotides for DNA synthesis. Well-documented RR1 and RR2 genes have been reported in the genomes of three GVs and 10 distinct Group II and a single Group I (OpMNPV) NPVs [79]. Two different RR2 genes have been reported for LdMNPV [88]. Based on the phylogeny of

baculovirus RR1 genes, it is postulated that two different capture events resulted in baculoviruses obtaining this gene [89]. For the OpMNPV and LdMNPV RR1 gene lineage, the source was a bacterium, whereas the other lineage (e.g., Spodoptera exigua MNPV (SeMNPV)) appears to have been derived from a eukaryote, most likely an insect. The two RR2 genes from LdMNPV appear to be derived independently, one from each different source rather than via gene duplication. No enzymology has been described for baculovirus RR and it is not known whether they have enzymatic activity, or how they integrate with or substitute for the homologous host enzymes.

How are baculovirus genomes replicated?

Whereas some genes have been identified that are required for transient DNA synthesis and major advances have been made in understanding the function of most of these genes, it is still not clear how baculovirus genomes are replicated. Evidence suggests that baculovirus replication results in DNA that is larger than unit length genomes. This DNA could be produced by rolling circle replication, DNA recombination, or by a combination of both these processes. The evidence for these two processes is described below.

Rolling circle replication. It was originally suggested that baculovirus genomes replicate by a rolling circle type mechanism because plasmids that were replicated in the transient system showed a ladder of multiples of unit length-sized DNA fragments when they were partially digested by a restriction enzyme with a single site in the plasmid [7]. It is not clear how closely the plasmid reflects the viral genome replication because of its much smaller size and minimal complexity. However, evidence has been presented suggesting that AcMNPV might replicate its genome in this manner [8]. Furthermore, it was found that plasmid DNA, when cotransfected with AcMNPV DNA, replicated to become high-molecular weight concatemers, some of which were integrated at a variety of locations in the viral genome. This was interpreted to suggest that both rolling circle replication and recombination may be involved in baculovirus DNA replication [5]. Further evidence will be described below that seems to support that larger than genome size DNA could be generated via DNA recombination.

Recombination-dependent replication. Baculovirus replication induces a highly recombinogenic state [45, 46, 90, 91], and this contributed to their development as expression vectors because foreign genes could be so readily incorporated into the genomes by homologous recombination. Recombination-dependent replication is a complex yet common mode by which many viruses replicate their DNA. It is unclear why systems have evolved this mode of replication, but evidence for it playing major roles in genome replication is found throughout DNA viruses with large genomes. T4 phage begins DNA replication in an origin-dependent manner and then switches to a recombination-dependent mode [92]. Lambda phage also provides an important model for recombination-dependent replication. Lambda DNA replication initially is of the theta type in which DNA is synthesized bi-directionally from a replication origin initially producing a bubble-like structure that progresses to resemble the Greek letter theta. However, later in infection, concatemers are generated by either rolling circle replication or recombination. Lambda encodes a recombination system called the Red system after mutations that were found to be *re*combination *d*efective. This system includes *red* α, β and γ that encode an exonuclease, an SSB, and an inhibitor of a host recombination system, respectively. RED α, the exonuclease, digests DNA in a 5' to 3' direction thereby generating 3' overhangs that anneal with complementary strands or invade homologous double strands; RED β the SSB, facilitates annealing of DNA strands , [93]. In addition, RED α and β interact forming heterodimers. There is a complex interaction between the host and phage recombination systems. A lambda-type phage, P22 is dependent on recombination and if both the host and phage recombination systems are inactivated, the phage will not replicate. Viability can be restored by the incorporation of the Red system into P22 [94].

It is thought that the replication of herpes virus genomes is recombination-dependent. DNA isolated from herpes simplex virus 1 (HSV-1) infected cells has a nonlinear, apparently branched, structure, and much of it will not enter a pulsed-field gel even after digestion with a restriction enzyme that cuts at a single site in the genome. In addition, SV40 DNA normally employs theta replication, but yields complex high MW DNA resembling HSV-1 DNA when replicated in an HSV-1 dependent system [95].

HSV-1 encodes a gene (UL12) homologous to the lambda *red* α exonuclease. The HSV-1 exonuclease also interacts with an SSB (UL29) [96]. The two HSV-1 proteins facilitate strand exchange in a manner similar to the lambda Red system [97]. When the HSV-1 exonuclease gene is inactivated, the production of infectious virions is severely compromised [98-100].

Similar to herpes virus, baculoviruses produce high molecular weight DNA that fails to enter a pulsed-field gel. When digested with an enzyme that cuts at a single site in the viral genome, much of the DNA is retained in the well. Also similar to herpes viruses, when SV40 is replicated in a baculovirus system, complex, high-molecular weight DNA is produced rather than products of theta replication [101]. In addition, all baculoviruses encode a homolog of the lambda *red* α exonuclease called alkaline nuclease (AN). It forms a stable complex with an SSB, LEF-3, and possesses both a 5'->3' exonuclease and endonuclease activity [17, 18]. LEF-3 has been shown to facilitate strand exchange in vitro [21]. These activities are consistent with the AN-LEF-3 complex being involved in DNA recombination. An AcMNPV bacmid with the AN gene deleted did not produce infectious virions after transfection into Sf9 cells. Also, although DNA replication levels appeared to be normal, much of the DNA generated appeared to be significantly smaller than observed in the control. These data demonstrated that *an* is an essential baculovirus gene [102] and suggested that it may be involved in the generation of larger than genome length fragments consistent with its role in a recombination system [9].

In summary, replication of baculovirus DNA has properties similar to other systems. These include the generation of what appear to be structurally complex DNA molecules that are larger than genome length, and the expression of two proteins, an exonuclease and an SSB, that interact with one another and have the properties of a system that is involved in DNA recombination in phage and other eukaryotic viruses. Furthermore, in viruses from phage to herpes viruses, homologs of the exonuclease are present and in all cases they interact with an SSB.

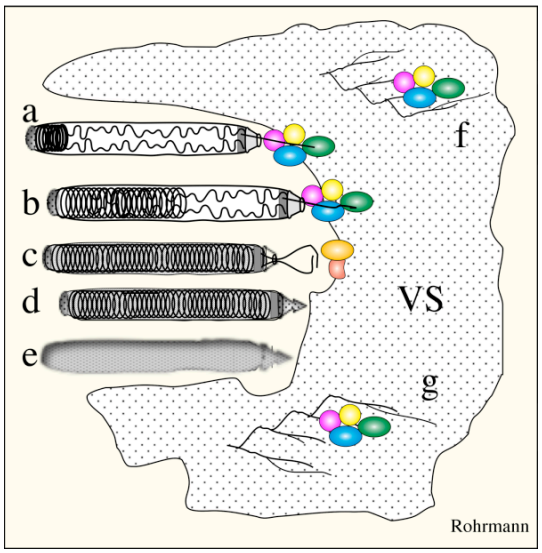

Fig. 5.2 Theoretical diagram of different stages of DNA replication and packaging. Shown is replication coordinated with packaging (a and b) and DNA replication that is independent of packaging showing extensive recombination (f,g). The large stippled area is the virogenic stroma (VS). The circularization of the genome by recombination is also indicated (c). Mature virions are represented by (d and e).

Implications of recombination-dependent replication: Multiple replication origins, a covalently closed circular genome, and multiple nucleocapsids per envelope

If recombination plays a major role in baculovirus DNA replication, it could explain several features unique to baculoviruses. These could include the possible use of multiple replication origins. Such origins could greatly amplify the amount of viral DNA that can be produced in a given time. The constraints of identifying and initiating replication at a specific site would be avoided, and replication could originate simultaneously at a number of sites. Upon recombination, molecules destined to become genomes would be produced that could begin or end anywhere as long as they were greater than genome length. In addition, the production of covalently closed circular DNA could be mediated by a final recombination event in which two homologous areas near the ends of a greater than unit length linear replication intermediate are joined (Fig. 5.2). Whereas

insect cells likely have extensive DNA recombination and repair systems, this final event in baculovirus DNA processing may be crucial for the production of an infectious virus. It is also possible that the propensity for some lepidopteran viruses to produce multiple nucleocapsids in a single envelope may facilitate recombination and repair of those that might be damaged [103]. Such damage could occur during their production, after they are released, or during infection in the midgut or within cells.

Processing and packaging of genome-size DNA

Although recombination based replication appears to solve several problems that are confronted by the replication of baculovirus genomes, recombination in combination with secondary initiation of replication might result in complex branched structures that would likely need to be resolved into covalently closed circular genomes of unit length before they could be packaged. The mechanism leading to the resolution of these structures is not clear. In addition to the extensive effects that alkaline nuclease and LEF-3 may have on genome processing, another protein, VLF-1 may also be involved.

Very late factor 1 (VLF-1) (Ac77). VLF-1 has been implicated in genome processing although its exact role is not clear. It is a member of the lambda integrase (Int) family of proteins. Integrases are a large group of site-specific DNA recombinases that catalyze DNA rearrangements and are found in a variety of organisms including viruses where they are involved in the integration and excision of viral genomes and decatenation of newly replicated chromosomes. A feature of these enzymes is that a conserved tyrosine forms a covalent link with DNA during the cleavage process. VLF-1 homologs are found in all sequenced baculovirus genomes suggesting that it plays a critical role in baculovirus biology. VLF-1 likely has two different functions in baculovirus biology. It is involved in the hyperexpression of very late genes [104] (see Chapter 6) and it also may have another function, independent of transcription, involved in genome processing.

An AcMNPV bacmid with *vlf-1* deleted appears to have normal levels of DNA replication, but fails to produce infectious virus. Aberrant tube-like capsids are produced by this mutant and they appear to lack DNA. Furthermore, viral DNA in this mutant appeared to be completely accessible to DNAse [105]. In contrast, other mutants with defective capsid production (e.g., a deletion of alkaline nuclease) showed significant levels of DNase protection (~35%) (Vanarsdall and Rohrmann, unpublished) suggesting that VLF-1 is required for DNA protection. When the bacmid with *vlf-1* deleted was rescued with a copy of VLF-1 that carries a mutation of the highly conserved tyrosine (Y355F), it restored the production of nucleocapsids with a normal appearance, but they were not infectious. Furthermore, the mutant appeared to be defective in the movement of nucleocapsids out of the virogenic stroma, suggesting that without active VLF-1, a final virion maturation step was blocked, possibly leaving the capsids tethered to the virogenic stroma by incompletely processed, larger than unit length DNA. Finally, VLF-1 appears to localize to an end region of the nucleocapsid. Collectively, these results indicate that VLF-1 is required for normal capsid assembly and serves an essential function during the final stages of the DNA packaging process [105-107].

In addition to evidence suggesting that VLF-1 is required for both proper capsid and genome structure, VLF-1 is capable of binding to several types of DNA structures that may further suggest that it has a possible role in DNA processing. It shows high affinity binding to cruciform DNA that mimics a structure common to recombination intermediates. No binding was evident to single and double stranded structures, and very low binding was observed to Y-shaped forks [57]. These results are consistent with the involvement of VLF-1 in the processing of branched DNA molecules at the late stages of viral genome replication. The ability of VLF-1 to bind homologous region (hr) sequences was also examined and it was found to bind to hrs that form hairpin and H-shaped structures. Although VLF-1 is capable of binding to branched DNA structures, enzymatic activity (endonuclease and topoisomerase) was not detected, suggesting that enzymatic activity of VLF-1, if present, may depend on accessory cellular or viral factors.

DNA packaging and nucleocapsid assembly. Early in the infection, DNA replication may result in the rapid amplification of DNA sequences that are used as templates for the expression of late

genes that encode a variety of proteins, including those involved in capsid structure. However, once capsids begin to be assembled and the concentration of viral DNA increases, genome production may be coordinated with the packaging of DNA into preformed capsids. The preassembly of capsids has been suggested by electron microscopy [108] and is a common feature of some large ds DNA viruses. However, genes that affect DNA replication and processing also appear to affect nucleocapsid structure suggesting that DNA is an integral component of nucleocapsid structure and without it, the capsid itself cannot assemble properly. A good example is the VLF-1 knockouts described above that produce empty tube-like capsids. Similar defects are observed with a deletion of the DNA binding protein, DBP [43].

Packaging DNA into a preformed capsid is an energetically unfavorable process and requires an ATP-driven packaging motor. Packaging motor complexes include a channel for the insertion of the DNA into the capsid and a set of enzymes that can compress the genome through the channel into the capsid. In tailed bacteriophage, the channel is composed of a ring-shaped portal structure embedded in the capsid. This forms a point for the nucleation of DNA packaging enzymes. Genome packaging motors of dsDNA viruses are comprised of a pair of components that are not part of the virion structure; the larger component binds to the procapsid, while the smaller component binds to DNA [109, 110] [111]. The motor protein associated with genome packaging of lambda phage has an endonuclease activity indicating that it may be involved in both packaging and DNA processing [112].

DNA recognition can be involved in packaging in two ways: a specific sequence may be recognized to initiate DNA packaging, and it may contact the motor complex during packaging. The specific sequence would ensure that only viral DNA would be packaged. Upon packaging within the capsid, DNA is concentrated up to 100-fold, often as a spool of concentric rings, so that it becomes highly condensed to near liquid crystalline density [113]. Viruses with concatemeric precursor DNA use either a 'headful' mechanism to measure DNA incorporation or utilize specific recognition sequences that bracket a complete genome. The cleavage at these sequences results in the excision of a complete genome sequence. It has been suggested that the 'headful' mechanism may cause a conformational change in the portal region as the head becomes full thereby triggering a mechanism to terminate packaging.

Energy for packaging can be derived either from the hydrolysis of ATP or by an electrochemical potential (proton gradient) generated across a membrane. Motor proteins contain active sites that bind ATP and catalyze its cleavage to ADP and Pi, releasing energy that causes a conformation change in the protein thereby driving the motor. Each cleaved ATP molecule can result in a processive, ratchet type movement driving the DNA into the capsid. In T4, the motor has a packaging rate of 700-2000 bp/sec with a force of >60 pN, one of the most powerful motors documented [114]. If similar, it would take less than 5 min to package the AcMNPV genome.

A candidate for a motor protein that might be involved in DNA packaging is Ac66. Homologs of *ac66* appear to be present in all baculoviruses. It is related to a variety of motor proteins including myosin heavy chain, a centromere protein, and Smc, a chromosome segregation ATPase that is involved in cell division. It was found to be associated with AcMNPV and HearNPV ODV [83, 115] However, an *ac66* knockout bacmid, although not viable, appeared to be normal and produced nucleocapsids with an electron dense core suggesting that they contained DNA [116]. It has been noted that during infection, actin that is normally in the cytoplasm, is transported to the nucleus [117] and interacts with structural components of the virion [118]. In addition, reagents that block actin polymerization may result in the production of aberrant capsids that appear to lack DNA [119]. Collectively, these observations indicate that components of the cytoskeleton could be involved in the insertion of DNA into nucleocapsids.

Coordinating DNA replication and packaging: A process for avoiding DNA recombination? A common feature of investigations that identify proteins associated with ODV is the presence of some proteins involved in DNA replication [83, 115]. This association may reflect remnants of macromolecular complexes that are 'frozen' in association with nucleocapsids by the occlusion process. Therefore, the presence of these replication-associated proteins with the nucleocapsid could reflect a highly coordinated set of reactions, including DNA synthesis, processing, and packaging in close proximity to the nucleocapsids. The insertion of DNA into

nucleocapsids as it is synthesized could protect the partially packaged DNA from strand invasion or nuclease attack. In addition, it is possible that DNA replication is partitioned such that DNA destined for genome production is highly coordinated with packaging to prevent extensive recombination. However, much of the DNA could be free to recombine and be destined to become templates for gene expression particularly for very late hyperexpressed genes that are transcribed after genomic DNA is packaged and inaccessible to transcription (Fig. 5.3). This is a theory, but in one experiment it was observed that late in infection upwards of half the DNA in a cell is DNAse sensitive suggesting that it was not packaged into nucleocapsids [105]. Therefore, if this DNA is accessible to the baculovirus RNA polymerase, it could provide a high copy number of very late genes to serve as templates for mRNA synthesis.

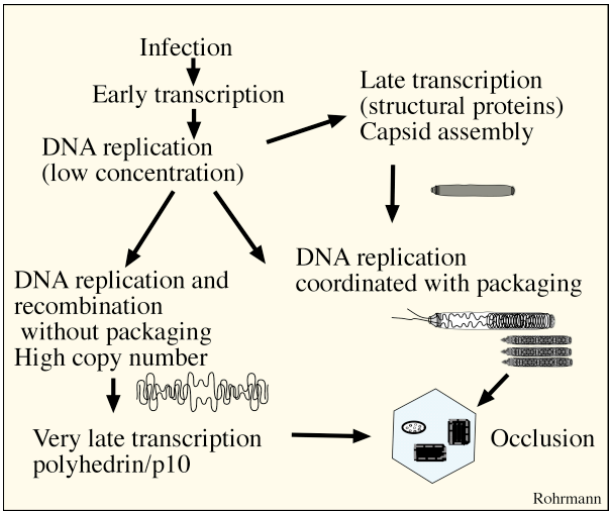

Fig. 5.3 Hypothetical diagram for roles of two types of baculovirus DNA; genomic DNA that is packaged and DNA that is not packaged and is essential for very late transcription.

More unanswered questions

The lack of genome isomerization. Similar to baculoviruses, herpes simplex virus I (HSV-1) and other herpes viruses are highly recombinogenic (reviewed in [97, 120]). During HSV-1 replication, intra-genomic inversions between two repeated elements within the genome results in a population of four different genome isomers. Since most baculovirus genomes appear to be punctuated with homologous repeated sequences that are distributed throughout their genomes (e.g. AcMNPV has 8 *hrs*, see above and Fig. 4.2) it is surprising that their genomes are replicated with such fidelity. It is clear that inversions do occur as there are examples of inversions bracketed by *hrs* between different viruses. For example, AcMNPV orfs 1-10 are bordered by *hr*1 and *hr*1a and are inverted relative to the homologous sequences in the *Orgyia pseudotsugata* MNPV genome [121]. However, there is no evidence that major populations of isomers are packaged into virions during normal virus replication similar to what occurs in herpes viruses. Restriction enzyme digestion of baculovirus genomic DNA results in a single characteristic pattern for each enzyme and that pattern conforms to the sequence of the genome. Since one might expect that *hr* inversions and other forms of recombination between these elements would be common during baculovirus replication, a mechanism must exist to either minimize these events or to eliminate such recombinants from the genome population. As described above, this could involve the partitioning of DNA replication such that DNA destined to become virion genomes is packaged as it is synthesized, whereas other DNA destined for use as templates for transcription is synthesized in a less coordinated manner and is subject to high levels of recombination.

Nucleocapsid length/genome size. Another major unanswered question involves the parameters that determine the length of the capsid and the size of the DNA molecule that is packaged. An examination of capsids associated with defective viral genomes suggested that capsid length may be flexible in response to genome size [122]. If capsids are both preassembled and can vary in length, it would suggest that they can be expanded or reduced in response to the size of the genome as part of the packaging process. The facility with which baculoviruses can be engineered to contain additional genetic material could also indicate that a unit size capsid may have some flexibility in the length of DNA that can be accommodated. How the virus senses that a genome is complete and terminates the encapsidation process remains to be determined.

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Chapter 6

Baculovirus late transcription

Activation of baculovirus late genes

A common feature of many viruses is the division of transcription into early and late categories. These divisions may be further separated into immediate early, early, late and very late. The separation between early and late transcription is always the onset of DNA replication. Despite this being a common feature of viruses, the mechanism underlying the linkage of DNA replication to the activation of late gene transcription is unclear. Two major features of newly replicated DNA may be involved in this process. Immediately after DNA replication there may be a transient period when proteins are not bound to the DNA and this might expose late promoters and facilitate their activation. In poxviruses, a set of transcriptional activators that facilitate the expression of late genes are expressed from newly replicated (naked) DNA [1]. In baculoviruses, the generation of DNA free of proteins might facilitate the activation of late promoters until p6.9 or other DNA binding proteins accumulate to such a level that all viral DNA is coated and transcription is inhibited. Another major feature of DNA in the process of being replicated is the numerous nicks and RNA-DNA junctions generated by the synthesis of Okazaki fragments during lagging strand synthesis. In viruses, e.g., T-4, it has been suggested that the unligated junctions of Okazaki fragments may serve as transient enhancers of late transcription by acting as loading sites for late gene activators that move along the DNA until late promoters are recognized [2]. Once DNA replication is completed, these loading sites for late gene activators might be eliminated by the removal of the RNA primers and ligation of the lagging strand thereby terminating late transcription. In baculoviruses essential activators of late gene transcription have not been identified in in vitro analyses, however, several additional factors have been characterized in transient assays that may be essential for late transcription (see below).

Baculovirus late promoter elements

Initially, because the sequence of the AcMNPV polyhedrin gene region revealed a conventional-appearing TATA sequence upstream of the reading frame, it was suggested that baculovirus late genes were transcribed using a conventional RNA polymerase II promoter [3]. However, the OpMNPV polyhedrin gene lacked this sequence and the mapping of its mRNA start site indicated that it initiated at an ATAAG sequence. When several other baculovirus late genes were sequenced [4-7], they were all found to have this sequence. This led to the proposal that ATAAG was the baculovirus late promoter sequence and that mRNA initiated within this sequence [8]. Subsequently, this was demonstrated experimentally [9] and eventually it was determined that the core sequence is normally ATAAG, GTAAG, or TTAAG, but that CTAAG is apparently not used. A survey of predicted baculovirus promoter sequences was conducted on 26 baculovirus genomes [10] (see Chapter 5). The purpose of this investigation was to determine whether baculovirus promoter elements could be correlated with their position relative to the ATG translation initiation codons. They found a number of clear correlations. There was a strong correlation of TAAG sequences upstream of the ATG, particularly when combined with a TATA early promoter, e.g. TATAAGG. These were about 7 times as frequent in the upstream location and reflect genes that are transcribed by both the host RNA polymerase II and the baculovirus RNA polymerase and are expressed both early and late in infection. TAAG sequences were almost 6 times more prevalent in the promoter (upstream) region. Of the TAAG sequences, ATAAG and GTAAG sequences were most prevalent, followed by TTAAG. In contrast, CTAAG, which does not appear to function as a late promoter element, was only slightly more prevalent in the upstream promoter regions.

An implication of the novel promoter and mRNA start site of late and very late baculovirus genes is that their transcripts would all begin with the

sequence UAAG. Whether this serves as a signal for selective sorting or transport of these mRNAs remains to be determined.

Insect virus RNA polymerases and occlusion body protein hyperexpression

Baculoviruses encode a novel RNA polymerase composed of four subunits that transcribes late and very late genes and that recognizes the unique promoter consensus sequence described above. It is not clear why a virus that replicates in the nucleus would encode its own RNA polymerase, since many such viruses depend on exploiting the host enzyme for transcribing all their genes. The remarkable ability of baculoviruses to hyper express very late genes might be considered the impetus for the evolution of a system that is independent of host transcription. Although several families of cytoplasmic RNA and DNA viruses, such as the Poxviridae and Reoviridae, encode their own RNA polymerases, most lineages of these viruses do not hyper express genes. However, both these families have occluded genera, the cypoviruses and entomopox viruses respectively, that are pathogenic for insects and express occlusion body proteins at very high levels reminiscent of baculovirus very late genes, e.g., entomopox spheroidin, can comprise 30-40% of total protein [11]. These viruses are able to accomplish this utilizing RNA polymerases related to those of other members of their viral family that do not hyper express genes, indicating that the evolution of the polymerase might not be specifically linked to its ability to hyper express genes. However, the independence of transcription from the host cell RNA polymerase could have facilitated the evolution of the extraordinary levels of gene expression of the polymerases of the occluded viruses. In the case of baculoviruses, the independence from the host enzyme may have initially provided regulatory advantages, such as independence from low levels of RNA transcription factors in nondividing cells. This could have provided the enzymatic platform for the evolution of their ability to hyper express very late genes involved in occlusion body formation. The combination of factors that likely influence high levels of baculovirus gene expression are discussed in detail in Chapter 10.

The baculovirus RNA polymerase

Hints of the presence of a novel baculovirus RNA polymerase had been suggested by the observation that the expression of baculovirus late genes was α-amanitin resistant [12, 13]. Alpha-amanitin is a fungal toxin that inhibits RNA polymerase II and hence mRNA synthesis at very low concentrations. The resistance to α-amanitin, along with the observation that baculovirus late genes employed a novel late promoter element, suggested that there was a distinct RNA polymerase involved in the expression of baculovirus late genes. Using a transient assay system that was dependent upon transcription from a late promoter element to express a reporter gene led to the identification of baculovirus genes that are involved in late gene transcription [14, 15]. This assay eventually implicated 19 genes in late transcription, however since the assay was dependent upon DNA replication, it included genes that were involved in that process. The DNA replication genes were identified using a separate assay (see Chapter 5), and the genes involved in late transcription were inferred by subtracting the replication genes. Subsequently, a protein complex of about 560 kDa was isolated that was able to support in vitro transcription from late promoter-containing DNA templates. This complex includes late expression factors (LEF) -4, -8, -9, and p47 with predicted molecular masses of 54, 102, 55 and 47 kDa, respectively. The combined predicted molecular mass of these four proteins is about 260 kDa. Because they appear to be present in equimolar amounts, it was suggested that the baculovirus RNA polymerase complex contains two molecules of each peptide [16]. The significance of this is unclear, as such a subunit composition has not be reported for other RNA polymerases. Homologs of these gene products are present in all baculovirus genomes that have been sequenced. Two of these subunits (LEF-8 and -9) have significant levels of similarity to the two largest subunits found in bacterial and eukaryotic polymerases, respectively (Fig. 6.1). These are called β and β' subunits, but they can vary greatly in size and consequently their size may be independent of their lineage.

LEF 9 contains a 7-amino acid motif (NTDCDGD) similar to the Mg++ binding sequence (NADFDGD) found in the catalytic center in the large RNA polymerase subunits of DNA-dependent RNA polymerases [17] (Fig. 6.1). The D residues are critical components of the

sequences because they coordinate the binding of Mg++ that is necessary for the activity of the polymerase. These three residues are conserved in all the orthologous large subunit sequences. AcMNPV LEF-9, at 516 amino acids, is much smaller than the other large RNA polymerase subunits, e.g., that of *Drosophila* is almost 1900 amino acids. Alignments indicate that LEF-9 is most closely related to the N-terminal 60% of the larger orthologous subunits. However, the homology is very low and other domains found conserved in this subunit from bacteria through eukaryotes and vaccinia virus [18] have not been reported in the baculovirus protein.

LEF-8 shows limited homology with the second largest subunit of a number of other organisms at the conserved 13-amino acid sequence GDKXX(s/g)RHG(q/n)KG(v/i/t). This homology is shown in Fig. 6.1 and compared to the orthologous *D. melanogaster* subunit and has been proposed to be part of the catalytic site [19, 20] (Fig. 6.2). The third subunit, P47, was originally identified as the site of a ts mutation that caused a defect in late gene expression [21, 22]. It has not been shown to be related to RNA polymerase subunits from other organisms. HHpred [23] is a program that detects protein homology by comparison of low levels of alignment (below 20%) with proteins domains from the SCOP (Structural Classification of Protein) database which contains all proteins of known structure and that is organized based on their structure, function and evolutionary relationships. When p47 was examined using this program, one of the 10 best matches was to an alpha subunit of *E. coli* RNA polymerase Fig. 6.1. These data indicated a 9% probability with 43% identity over 14 amino acids. In addition, when a p47 from *Neodiprion sertifer* NPV was tested, 4 of the 10 best matches, including the best match, were α subunits of bacterial RNA polymerases with probabilities of about 10%. In all instances the sequences showed similarity in the same domain (Fig. 6.1). The significance of this similarity needs to be determined. In contrast, this program indicated that LEF-8 had similarity with a high level of probability (97.4%) to the large subunit of RNA polymerase II of *Saccharomyces cerevisiae* (23 % identity over almost 200 amino acids) and LEF-9 showed 80% probability of relatedness to a large RNA polymerase subunit of *Sulfolobus solfataricus* (24% identity over about 70 amino acids). LEF-4 is an RNA capping enzyme (see below) [24, 25].

The categories of RNA polymerases

There are four major lineages of RNA polymerases that appear to be unrelated. These include i) RNA-dependent RNA polymerases of RNA viruses, ii) the polymerases of some phage such as T7, iii) primases that produce short RNA transcripts for priming DNA replication, and iv) the DNA-dependent RNA polymerases of eubacteria, archaea, and eukaryotic cells. The DNA-dependent RNA polymerases of bacteria and eukaryotes are highly conserved enzymes

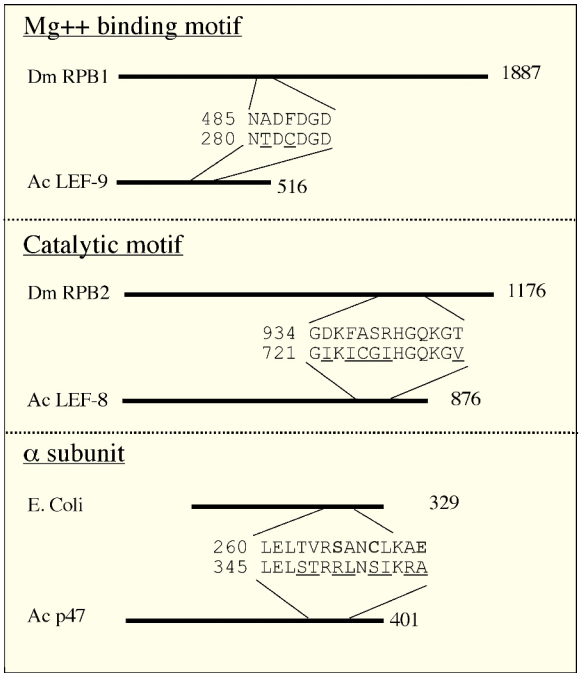

Fig. 6.1. Homology of baculovirus RNA polymerase subunits. The similarity of the LEF-8 and -9 subunits to the two largest RNA polymerase subunits of *Drosophila melanogaster* RNA polymerase II is shown. In addition, the relatedness of P47 to the alpha subunit of *E. coli* RNA polymerase as determined by HHpred is indicated (see text). The numbers at the end of the lines indicate the size of each protein in amino acids. The numbers before the sequences indicates the location of the domain within the sequence. The underlined amino acids are not conserved.

and are composed in most organisms of 5 to 15 subunits. Four of these correspond to α, β, β' and ω subunits of bacteria and form the conserved core and orthologs are present in all the cellular enzymes (Fig. 6.2). The β and β' subunits interact with each other and the active site is formed by their interface ([26] and references therein). The β' subunit contains a catalytic site and has three invariant aspartate residues that interact with a Mg2+ ion and the α-phosphate of the NTP during polymerization [27]. Subunits related to the β and β' polypeptides are also encoded by the genomes of a number of families of large DNA viruses that are pathogenic for eukaryotes [28]. The α subunit is involved in the initiation of RNA polymerase assembly and sequence specific protein-DNA interactions that result in promoter recognition, and is also a target for transcriptional activation [29]. Two identical α subunits are present in bacterial RNA polymerase and four homologs of the α subunit are found in the polymerases of archaea and eukarya (Fig. 6.2). Surprisingly a homolog of the α subunit has not been described for the vaccinia RNA polymerase [30]. As described above p47 shows sequence similarity in one region to bacterial α subunits.

In addition to the subunits that comprise the RNA polymerase, transcription of genes often requires other additional factors. Bacterial RNA polymerases recognize specific promoter elements by interacting with sigma factors that specify transcription of specific categories of genes. However, transcription from the eukaryotic RNA polymerases involves a variety of transcription factors, some of which are composed of multiple subunits. Perhaps as a consequence of the size and complexity of eukaryotic polymerases, the promoters to which the polymerases bind and the location of the transcription start sites are separated by 30 bases or more. Although not as complex, the promoter and start site region of bacterial polymerases encompasses up to 35 bp. Finally, the RNA polymerases of phage such as T7 are composed of a single subunit unrelated to those described above, and have a compact polymerase binding RNA start site of about 20-bp [31]. In contrast, as described above, both the baculovirus RNA polymerase promoter and mRNA start site are very compact and located at TAAG sequences.

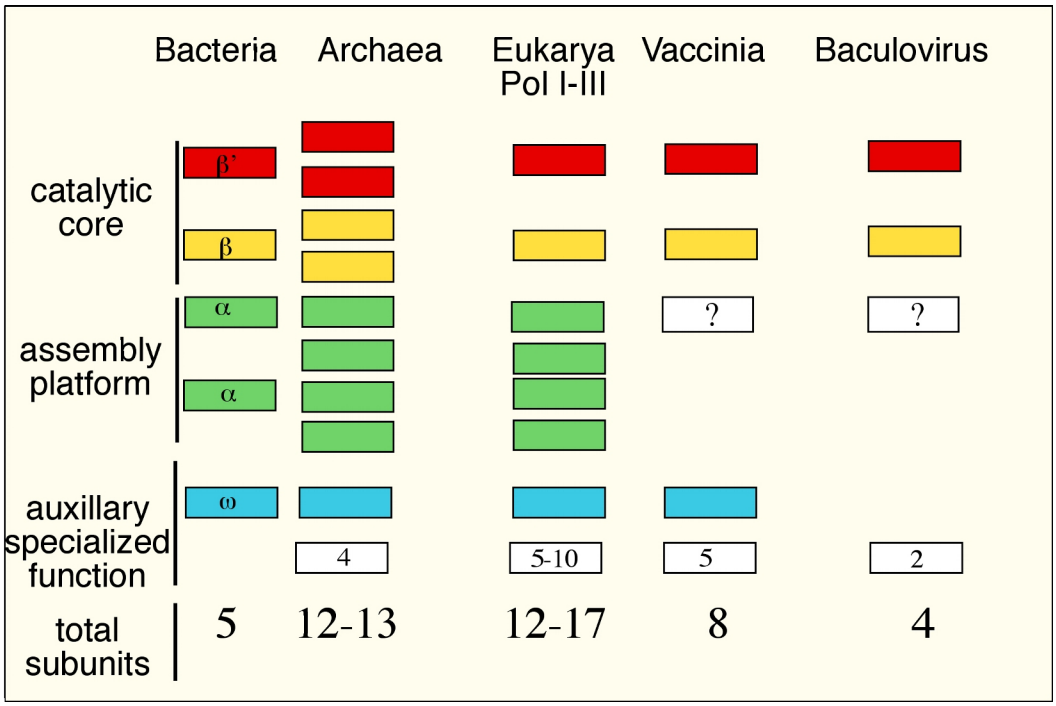

Fig. 6.2. Relationships of RNA polymerase subunits. The large subunits, β' and β, in some archaea have undergone fission and are present as either 3 or 4 components. The subunits are color-coded to indicate similar function. This figure is based on the data of [26, 30, 32, 74, 75]. For description of baculovirus homology, see text.

The relationship of baculovirus RNA polymerase to other RNA polymerases

Although there is homology to the β and β' subunits of RNA polymerase, as summarized above the degree of relatedness is very limited making it difficult to determine the lineage of the baculovirus polymerase. In addition, no other RNA polymerase appears to have a promoter similar in sequence to that of late baculovirus genes (see below). The initiator sequences of RNA polymerase II (Chapter 4) are similar in that transcription initiates at the promoter sequence. However, the extent of the interaction of the baculovirus RNA polymerase with promoter sequences has not been determined, possibly because the complex is so unstable that it cannot be footprinted using conventional techniques. Analysis of the RNA polymerase subunits of bacteria, archaea and eukaryotes found that they almost all encode four subunits related to the bacterial β and β', α and ω [26]. The RNA polymerase of the poxvirus, vaccinia, is similar to those of eukarya as it has a complex subunit composition with at least eight subunits, seven of which appear to be orthologs of RNA polymerase II subunits β and β' plus RPB 5, 6, 7, and 10 and the transcription factor SII [30, 32] (Fig. 6.2). The baculovirus RNA polymerase is distinct from these other major DNA dependent RNA polymerases in that it is composed of four subunits. Since the catalytic cleft is located on the two largest peptides, and baculoviruses possess orthologs of these two subunits, the basic enzyme core is retained. However, based on current analyses, the phylogenetic origin of the baculovirus RNA polymerase is unclear.

LEF-4, an RNA capping enzyme

The 5' RNA cap is a novel feature of mRNAs from eukaryotic cells and viruses that is not present in bacteria and archaea, because they lack the necessary enzymes for its synthesis. The cap is added to the 5' end of mRNA and consists of a guanine nucleotide linked to the mRNA with a 5' to 5' triphosphate linkage. The guanosine in methylated at the N-7 position by an enzyme called methyl transferase. It can be further modified by an additional methylation of the 2' hydroxyl groups of the ribose sugars at the 5' end of the mRNA (Fig. 6.3). This results in the RNA resembling the 3' end of an RNA molecule-- the 5' carbon of the cap ribose is blocked whereas the 3' position is free. Capping involves a RNA 5' triphosphatase that removes the terminal phosphate of the RNA, a guanylyl transferase to add the guanine, and two different methlyases. The first two activities are present in LEF-4 and are also present on a single protein located at the N- and C-termini, respectively, in metazoans and plants. Although having similar activity, LEF-4 is unrelated to this category of capping enzyme, but is a member of the metal dependent group of capping enzymes found in fungi and protozoa [24]. The enzymes that are involved in the two methylase reactions are not known, although AcMNPV does encode a methyltransferase homolog (Ac69) that would be capable of carrying out the second methylase reaction (see below).

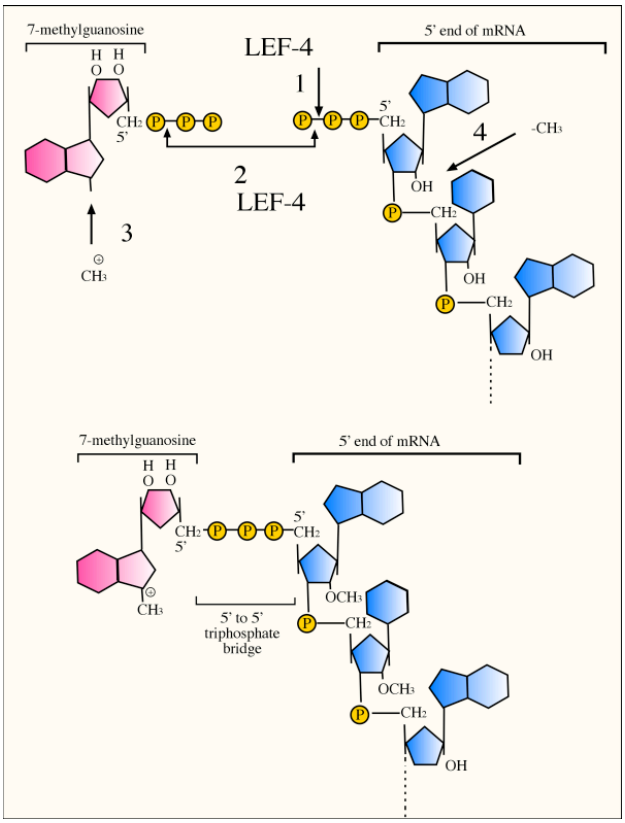

Fig. 6.3. Capping baculovirus mRNA. The four modifications that occur during capping are summarized in the following steps: 1; LEF-4 has the potential to dephosphorylate the 5' end of mRNA; 2, and to transfer guanosine to the 5' end of the mRNA; 3, the guanosine is methylated-- enzyme is unknown; 4. The 2' OH group of the terminal A is methylated by an unknown methytransferase. The final structure is shown at the bottom.

The 5' cap structure is thought to have several major roles associated with mRNA function. These include: i) nuclear export: it binds a protein that is recognized by the nuclear pore complex and is exported; ii) protection from exoribonucleases: this can be important because the export process can take significant amounts of time during which the RNA is subjected to exonuclease exposure; iii) promotion of translation: the cap serves to recruit initiation factors, which in turn recruit ribosomes [33]. Capping can also be involved in intron excision; however, this may not be a major role in baculoviruses because of the limited amount of splicing that occurs.

Capping in eukaryotes involves an enzyme that associates with the highly repetitive carboxyl terminal domain (CTD) of the β' subunit of RNA polymerase II. Because the baculovirus polymerase lacks a similar domain, it is likely that it evolved to include the enzyme as part of the RNA polymerase complex. Similarly, vaccinia virus also lacks a CTD on its β' subunit and its capping enzyme is also associated with the RNA polymerase complex and capping occurs during transcription when the nascent RNA oligomer is about 30 nt long [34]; however, it does not appear to be an integral component of the polymerase enzyme.

Assuming that in vitro transcription reactions are free from exoribonuclease, it is not clear why LEF-4 is required for transcription in vitro assays. These assays monitor RNA transcripts that would not need to be capped in order to be detected. This suggests that LEF-4 may play a structural role in the organization of the polymerase subunits or it may have some other function. Furthermore, inactivation of both the LEF-4 RNA triphosphatase domain and the RNA triphosphatase encoded by Ac orf1, did not affect the viability of a double mutant virus. Therefore if capping is required in cultured cells, these data suggest that a host enzyme can carry out the triphosphatase reaction. In addition, the chemical composition of the baculovirus cap structure does not appear to be identical to the conventional motif [35]. However, variant cap structures have been described in at least one other virus [36]. Complicating an interpretation of the role of LEF-4 in capping is evidence indicating that the 5' untranslated region of p10 mRNA facilitates translation in a cap independent manner [37]. Since commonly used internal ribosome entry sites are apparently not active in certain insect cell systems, the p10 5' untranslated region has been used as a translational enhancer of uncapped mRNA in investigations employing insect cells [38].

Ac69, a methyltransferase (MTase)

Ac69 encodes a methyltransferase and it is found in all Group I NPV, about one-half the Group II NPV and in the NeseNPV genomes. Ac69 was found to stimulate late gene transcription in a transient assay [39]. The gene encodes a protein with RNA Cap (nucleoside-2′-O)-methyltransferase activity. AcMNPV, with a null mutation of the gene, replicated normally in cell culture [40]. Similar results were observed for a knockout of the homolog (Bm57) in BmNPV [41]. Therefore, it is not clear whether a host gene can carry out this function or the viral gene may not be necessary for viral replication in cell culture.

Ac38- a baculovirus decapping enzyme?

Ac38 is an ADP-ribose pyrophosphatase (ADPRase), a member of the subfamily of Nudix pyrophosphatases. Orthologs are found in all lepidopteran NPV and GV genomes. Proteins of the nudix superfamily are common in all organisms and have been reported in other viruses, including T4 bacteriophage, African swine fever virus (family : Asfarviridae), and poxvirus. An AcMNPV Ac38 deletion mutant was severely compromised and produced BV at 1% the level of wt [42]. Vaccina virus also encodes a nudix protein and it may be important in negatively regulating gene expression by acting as a decapping enzyme [43] by removing the 7-methylguanosine diphosphate. Deletion of the gene in vaccinia resulted in smaller plaque and low virus yield [44], similar to the *Ac38*-deleted AcMNPV.

In vitro transcription assays

It should be noted that much of the information regarding baculovirus RNA polymerase has been derived from in vitro transcription assays [16, 45] and these systems involve the addition of DNA

templates containing late promoter elements. Since this DNA is purified and presumably protein free, it is not clear how well these systems reflect late transcription in infected cells. Future analysis of transcription from templates complexed with proteins may yield information on additional proteins involved in the activation of late transcription. In addition, whereas the baculovirus RNA polymerase complex is limited to four proteins, a number of other proteins are required for late transcription in transient assays (see below).

Termination of late mRNAs

Early mRNAs are transcribed by the host RNA polymerase II and it is assumed that 3' processing is carried out by enzymes associated with this complex. It was also assumed that baculoviruses used the host 3' mRNA processing apparatus to cleave and polyadenylate late mRNAs downstream of the coding sequence. However, evidence has been presented that suggests that 3' ends may be formed by the presence of T-rich sequences that destabilize the late transcription complex [46] (Fig. 6.4). It is not clear how the ends are polyadenylated as this process is normally associated with 3' cleavage by enzymes associated with the CTD present on β' subunit of eukaryotic RNA polymerases. As indicated above, this structure is not present in the baculovirus polymerase.

Very late gene expression

Very late factor 1 (VLF-1, Ac77). A novel feature of baculoviruses is their ability to express genes at high levels very late in infection. This phenomenon has been exploited in the development of baculoviruses as expression vectors. Two highly expressed very late genes have been characterized, polyhedrin and p10. Polyhedrin is the occlusion body protein, whereas the role of p10 is not clear, although it appears to form fibrillar structures that may be involved in the assembly of the polyhedron envelope during the maturation of polyhedra [47] (see Fig. 2.2) and may influence the lysis of terminally infected nuclei [48]. Because these genes appear to be involved in polyhedron morphogenesis, which is a very late step in the baculovirus life cycle that occurs after virions destined for occlusion have been assembled, it is likely that they are transcribed from DNA that does not become packaged as virion genomes. Consequently, it remains accessible to the very late RNA polymerase complex. Both polyhedrin and p10 genes contain an A/T-rich sequence downstream of a late promoter sequence that is involved in their high level expression [49]. This sequence was called the 'burst sequence' because it caused a burst of transcription very late in infection. Very late expression factor 1 (VLF-1) was originally identified because it influences the hyperexpression of very late genes [50]. Subsequently, it was found that VLF-1 interacts with the burst sequence in gel shift assays [51] and the presence of this sequence stimulates the level of VLF-1. In vitro transcription assays suggest that VLF-1 can stimulate very late transcription about 10 fold. In addition, when the burst sequence is removed the level of stimulation is reduced about four fold [52] (Fig. 6.4). Homologs of VLF-1 are found in all sequenced baculovirus genomes and they belong to a family of proteins that includes lambda integrase. Integrases are a large group of site-specific DNA recombinases that catalyze DNA rearrangements and are found in a variety of organisms including viruses where they are involved in the integration and excision of viral genomes and decatenation of newly replicated chromosomes. Evidence suggests that VLF-1 may also be involved in the processing or packaging of baculovirus genomes (see Chapter 5).

LEF-2 (Ac6) and PK-1 (Ac10). In addition to VLF-1, LEF-2 (Ac6) appears to be involved in very late transcription. Although it is an essential replication gene where it apparently functions as a primase accessory factor (see Chapter 5), LEF-2 mutants of AcMNPV have been characterized that appear to affect very late transcription indicating that it may have roles in both replication and transcription [53]. In BmNPV, LEF-2 has also been shown to activate late transcription [54] independent of its influence on DNA replication. It has been suggested that Ac10, which encodes a serine/threonine kinase (called PK-1), also influences very late gene expression. Orthologs of Ac10 are found in all lepidopteran baculovirus genomes. The inhibition of PK-1 expression by either a ts mutation [55] or by DNAzyme technology causes a reduction in the expression from the polyhedrin

promoter [56]. PK-1 also may be associated with a very late transcription complex and be involved in the phosphorylation of LEF-8 [57].

In summary, it is likely that high levels of gene expression are influenced by several features of baculovirus biology. These include: i) the amplification of genes by DNA replication (see Chapter 5); ii) the shutoff of most late transcription, possibly by DNA binding proteins that coat the DNA and thereby make RNA polymerase available for very late transcription; iii) the efficiency of the late polymerase and VLF-1 in recognizing and initiating from very late promoter elements; iv) the efficiency of LEF-4 in capping the mRNA (see Fig. 6.3), and v) a possible role for LEF-2 and PK-1. As mentioned above, the 5' untranslated region of p10 mRNA appears to be capable of facilitating cap-independent translation, which may reduce the reliance of these transcripts on LEF-4 activity [37]. Other factors that might enhance translation of very late expressed mRNAs have not been identified. For a detailed discussion of the factors influencing very late transcription, see Chapter 10.

Other genes involved in late transcription

With the removal of the six essential replication genes and the four genes encoding the late RNA polymerase complex from the total of 19 genes that have been implicated in influencing late gene transcription [15], leaves the roles of nine genes unexplained. These genes are required for or stimulatory for late transcription in the transient assay system. One of these genes, p35 blocks apoptosis and therefore, by preserving the viability of cells, promotes both viral DNA replication and late transcription. LEF-7 is stimulatory for DNA replication, and although LEF-11 was not identified as being involved in DNA replication in the transient assay system, a knockout construct is defective in DNA synthesis (see Chapter 5). IE-2 may influence late transcription because of its role in the activation of early transcription (see Chapter 4). Therefore, LEF-7 and IE-2 could affect late gene transcription by influencing DNA replication. This leaves five genes that may specifically influence late gene transcription. These are described below.

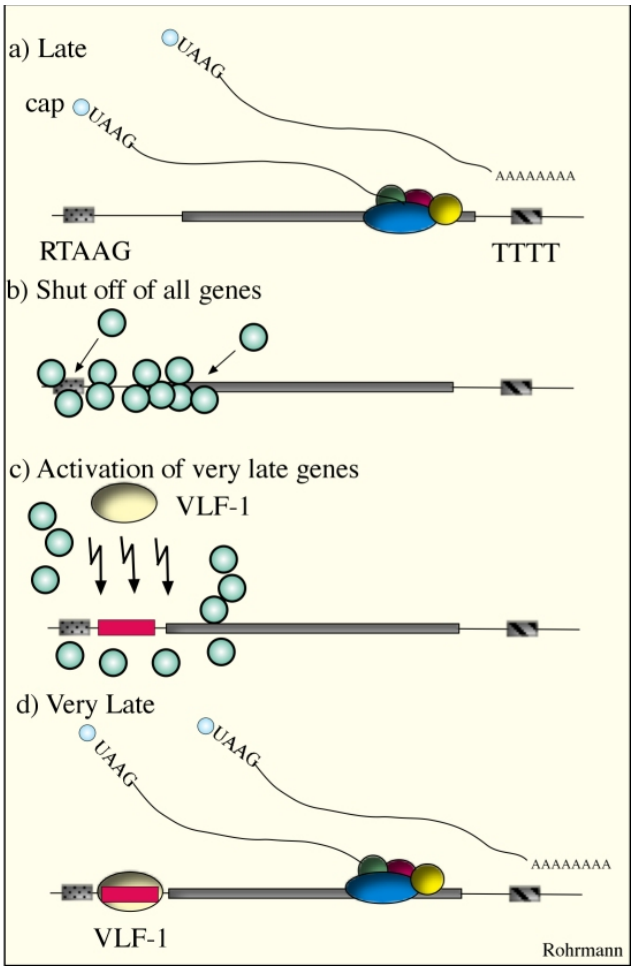

Fig. 6.4. Comparison of late and very late gene transcription. Shown is late gene transcription (a), and a hypothetical mechanism for the shutoff of late transcription (b), and activation of very late gene transcription involving VLF-1 interacting with the burst sequence (shown as a red rectangle) (c and d). Late transcription initiates within the late promoter element. Therefore all late messages likely begin with the sequence UAAG.

Lef-5 (Ac99), a homolog of TFIIS. Homologs of *lef-5* are found in all baculoviruses and are also present in nudiviruses [58]. LEF-5 was originally identified as being required for transient late gene transcription [59]. It was demonstrated to interact with itself and to contain a domain similar to that of the RNA polymerase II elongation factor TFIIS [60]. Subsequent investigations indicated that LEF-5, although highly stimulatory for in vitro transcription, did not enable the baculovirus polymerase to transit pause sites and it was concluded that it functions as an initiation factor,

rather than an elongation factor [61]. Deletion is probably lethal as interrupted mutants of this gene in BmNPV (Bm83) could not be isolated [62].

Lef-6 (Ac28), a possible mRNA export factor. Homologs of *lef-6* are found in the genomes of all lepidopteran NPVs and GVs. It was originally identified because it was required for transient transcription of late genes [63]. A bacmid deleted for lef-6 was infectious, but the infection was delayed and the titer was reduced to about 10% of wt. The major effect appeared to be reflected in a delay in the onset of late transcription [64]. LEF-6 is not particularly well conserved. Although AcMNPV and LdMNPV showed only 27% amino acid sequence identity, using the HHpred program described above, both showed over 65% probability of encoding a region that is related to the RNA binding domain of a factor called TAP that is involved in the export of mRNA out of nuclei through their interaction with nuclear pore proteins [65, 66]. If LEF-6 performs a similar function, it might compensate for the nuclear transport pathway if it is compromised by the infection. It might also be essential for the transport of the high levels of mRNA produced by very late genes.

Lef-10 (Ac53a). This orf was named Ac53a because it was not identified in the original AcMNPV genome sequence because it is a small orf encoding 78 aa and about half the coding region at the 3' end overlaps the 5' region of Ac 54. Homologs of lef-10 are found in all Group I and most Group II NPV and GV genomes. Lef-10 was originally identified because it was required for late gene expression [17]. It is likely an essential gene as an insertion mutant in the BmNPV homolog (Bm42a) could not be isolated [62].

Ac41 (Lef-12). Lef-12 is found in about half the Group I and Group II NPV genomes sequenced. Although 18 genes were originally identified as being involved in transient expression from a late promoter [14], when a set of these genes were individually cloned, they failed to support late transcription. Because of its close proximity to Ac 40 (p47), Ac41 (*lef-12*) had not been identified in the initial screen. It was subsequently demonstrated to be required for transient late gene transcription *in S. frugiperda* cells [15, 39], but not required for late transcription in *T. ni* cells [15]. Mutants with *lef-12* interrupted by insertional mutagenesis or by mutation of the ATG translation initiation codon were viable in both *S. frugiperda* and *T. ni* cells, although reduced yields of BV were observed (20-40% of wt) in both cell lines and the infection cycle appear to be slowed [67]. It was suggested that *lef-12* may be functionally redundant in the AcMNPV genome and therefore it is not essential for late transcription when the rest of the virus genome is present [67].

pp31 (39K -Ac36). Pp31 was originally identified because it contains an early promoter that is stimulated by IE-1 [68]. Homologs are present in all lepidopteran NPV and GV genomes. It is phosphorylated and localizes to the virogenic stroma of infected cells, and is capable of binding to DNA, but is not a virion structural protein [69]. Purified PP31 binds to single-stranded and double-stranded DNA with equal affinities and inhibited transcription in vitro [70]. Phosphorylation of PP31 is a dynamic process [71] and several basic regions appeared to be involved in nuclear localization and one of these regions is involved in DNA binding [72]. It was found to stimulate late gene transcription in a transient transcription assay [14]. Deletion of the *pp31* homolog in BmNPV (Bm27) resulted in virus that, although viable, showed a reduction in late gene transcription, a 100 fold reduction in BV production, and improper formation of the virogenic stroma [62]. Similar results were obtained for an AcMNPV bacmid deleted for *pp31* and it was observed that the deletion resulted in a significant decrease of the transcription several late genes that were examined [73]. It is not clear whether this gene acts directly on late transcription or may be a structural protein of the virogenic stroma and is required for the optimal organization of this structure and thereby indirectly influencing late transcription.

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Chapter 7

Baculovirus infection: The cell cycle and apoptosis

The cell cycle and apoptosis are interrelated. If a cell detects perturbations in different stages of its replicative cycle such as those caused by viral infection, the cell can be induced to undergo apoptosis. Because baculovirus replication causes major alterations in the cell cycle, it has evolved several mechanisms to interfere with apoptosis.

Baculovirus infection and the cell cycle

Cell division involves a cycle of defined stages during which DNA precursors and other cellular components are synthesized, chromosomes are duplicated and segregated to opposite poles of a cell during mitosis, followed by division of the cell. These stages in cell growth have been named gap or G phases (G1, G2, and G0), S for the synthetic phase, and M for the mitotic phase (Fig. 7.1). G1 follows mitosis during which the cell is prepared for DNA replication by the production of synthetic enzymes, S follows G1 and involves the DNA synthesis phase during which chromosomes are duplicated, G2 follows S and involves preparation for cell division which occurs in M or the mitosis phase. If a cell becomes dormant and is not in a replication cycle it is in stationary or G0 phase. The cell cycle is highly regulated and the orderly progression through each phase is critical for chromosome replication, separation, and production of daughter cells. The transitions between the different stages of the cycle are governed by the phosphorylation state of a number of proteins called cyclins. They are phosphorylated by cyclin dependent kinases (cdks) (Fig. 7.1). Progress through the cell cycle is also governed by molecular checkpoints that can prevent the continuation of the cell cycle at specific phases. Deregulation of the cell cycle can result in uncontrolled cell proliferation, a hallmark of cancer development. Consequently regulation of the cell cycle has been intensively investigated.

The cell cycle can be monitored experimentally by measuring the DNA content in each cell in a population by flow cytometry. In this process cells are fixed in ethanol, RNA is removed by treatment with RNase, and the DNA is stained with propidium iodide, and quantified for each cell by flow cytometry. Two major populations of cells are normally present, those in G1 phase that contain a set of diploid chromosomes, and those in G2 and M phase (G2/M), in which the chromosomes are doubled and have twice the DNA content of diploid cells. Normally, a diffuse population of cells in S phase is present and unless the cells have been synchronized, it is not particularly distinct.

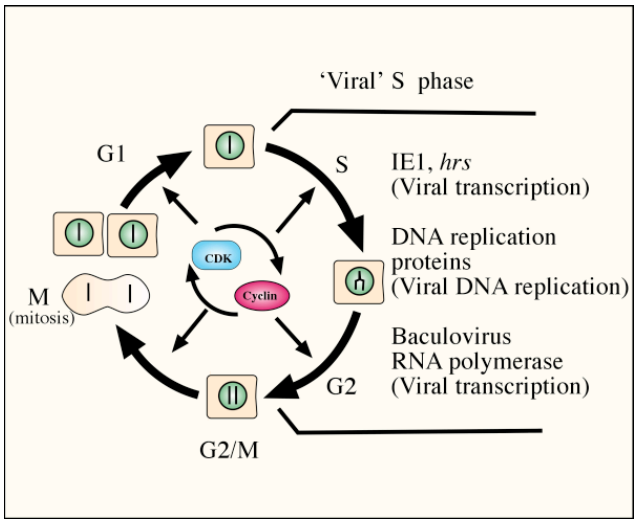

Fig. 7.1. The cell cycle is regulated by the phosphorylation state of different cyclin molecules. Baculovirus infection appears to block the cell cycle and prevents cells from undergoing mitosis. They likely induce a 'pseudo' S phase-like environment in which the virus transcriptional activator IE1, along with *hr* enhancer sequences preferentially activate early viral genes leading to DNA replication and the production of the baculovirus RNA polymerase.

Many viruses block the cell cycle [1]. This may seem logical, because it would block competition by a dividing cell for the cellular replicative apparatus and components and allow these to be directed towards viral replication. However,

blockage of the cell cycle can result in very low levels of enzymes required for transcription and DNA synthesis because they are no longer needed for cell division. For example, in some cells replicative DNA polymerases and other factors associated with DNA replication are amplified threefold or more during S-phase [2]. A number of DNA viruses do not encode the substrates required for DNA synthesis. When these viruses infect cells that are in interphase and are not undergoing cell division, there is a reduced availability of compounds necessary for DNA synthesis. Consequently viruses have devised several methods to induce nonreplicating cells to either enter S phase or to induce a pseudo S phase in which the cellular synthetic machinery is activated, allowing for virus replication independent of cell replication. Some viruses can induce cells to enter S phase by expressing proteins that inactivate a protein called Rb. It is named for the retinoblastoma susceptibility gene that was first implicated in cancer of the retina, a disease that usually occurs before the age of five in about 1 in every 20,000 children. In this tumor, the Rb protein is inactivated and that led to its discovery. Rb governs the G1/S check point by binding to a transcription factor (E2F). If Rb binds to certain viral proteins, it releases the transcription factor (E2F) that can then activate S phase. Normally Rb is regulated by its phosphorylation state–it binds to the S-phase activator when it is not phosphorylated. The inactivation of Rb can lead not only to the entry of the cell into S phase, but to unregulated cell growth and division, which is a characteristic feature of virus-induced tumors. Therefore, the byproduct of the necessity of the virus to induce S phase can be a tumor. This is especially true when the portion of the virus genome encoding the inhibitor of Rb integrates into the cell genome, thereby permanently deregulating the cell cycle. Examples of viruses capable of causing this mutation are tumor viruses, such as human papilloma, SV40, and adenovirus.

Another checkpoint governs the transition from G2 to M. It is thought that regulation of this transition is involved in monitoring for DNA damage. If DNA damage is detected, then progression into mitosis is inhibited and the cell may undergo apoptosis. This prevents the establishment of cells with defective DNA genomes. Many viruses appear to block the cell cycle at G2/M, thereby rendering the cells unable to divide [1]. This may be caused by the cell checkpoint monitoring systems that detect viral DNA replication and regards it as damaged cellular DNA and thereby stops the transition to metaphase. Concomitant with blocking the transition to M phase, a number of viruses appear to induce what is called a ‘pseudo’ S phase. Baculoviruses likely fall into this category as several reports have indicated that infection causes cells to be blocked at G2/M. One report found that most non synchronized cells were blocked at G2/M after AcMNPV infection [3]. In another report, synchronized Sf9 cells infected in G2/M were arrested in that stage, whereas those infected at G1 were arrested in S phase [4]. In yet another study it was reported that cells infected at G2/M, G1, or S phases, were arrested in the S or G2/M phase [5]. Investigations on infection with another virus, HaSNPV, in a different cell line, also resulted in a block at G2/M. Normally cyclin B1 is degraded at the onset of metaphase allowing progression through this phase, however in these cells it accumulates and remained at a high levels [6]. An AcMNPV protein (Ac144) was identified that may contribute to the blockage at G2/M [7].

The various observations regarding baculovirus infection and the cell cycle could be a reflection of the blockage of cellular DNA replication upon infection and the subsequent replication of viral DNA, which would obscure the phase of the host cell cycle. Whereas blocking the cell cycle may be necessary for diverting the cellular replicative machinery for virus replication, it is not understood how the cellular replicative apparatus is exploited by the virus. Clearly, however, for viruses that are dependent on host enzymatic pathways and synthetic machinery, the activity of these systems would either be preserved or elevated during the infection.

Viral synthetic pathways: Induction of a 'pseudo' or ‘viral’ S phase environment

Several factors likely contribute to the ability of baculoviruses to replicate independently of the cell cycle and produce a 'pseudo' S phase like environment, which might be more appropriately termed ‘viral’ S phase because of the components that the virus contributes to macromolecular synthesis. These include the ability of cells to selectively transcribe viral genes

through the transactivator IE1 in combination with *hr* enhancer sequences, the synthesis of a set of DNA replication proteins so that virus replication is independent of host proteins for DNA replication, and the production of an RNA polymerase so that they are independent of the host RNA polymerase II. These factors are summarized in Fig. 7.1. Another contributing factor would be the shut down of most host RNA polymerase II transcription [8]. In addition, there are likely a few other cellular pathways that are exploited by the virus for DNA synthesis. Hints of some of these are likely reflected in enzymes involved in biosynthetic pathways encoded by some, but not all baculoviruses. It is likely that there are advantages for the genes encoding these enzymes to be incorporated into the viral genome because then they would be under the regulatory control of the virus. However, in viruses lacking these genes, they likely remain dependent on the host pathways. An example of one such enzyme is ribonucleotide reductase (RR) that is encoded by a minority of baculoviruses and consists of two genes that encode a heterodimer composed of large and small subunits (RR1 and RR2, respectively). RR is involved in the catalysis of ribonucleotides to deoxyribonucleotides as a pathway for providing nucleotides for DNA synthesis. It is found in most Group II NPVs, a single Group 1 NPV (OpMNPV) and three of the ten (as of 2008) GV genomes that have been sequenced. Although it is not clear why some viruses encode this enzyme and others do not, it does suggest that it may be a required component for optimizing the cellular environment for viral replication and indicates that viruses that lack the enzyme are dependent on the host for this pathway. In all of the NPVs and one of the GVs encoding the RR genes, they are accompanied with a companion enzyme, dUTPase. One of the products of RR can be dUTP, which if incorporated into DNA, can be mutagenic. Consequently the presence of both RR and dUTPase suggests that the latter enzyme mitigates the mutagenic potential of dUTP, which is inactivated by dUTPase.

It has also been noted that many of the most prevalent promoter sequences present in baculovirus genomes are combinations of both early and late promoters (see Chapter 4) [9] such that the genes can be initially expressed by the host RNA polymerase II, but can also be expressed by the viral polymerase. This promoter arrangement would ensure that sufficient levels of the protein are present independent of host cell at both early and late times post-infection.

Apoptosis and baculovirus infections

Apoptosis or programmed cell death is a pathway that is thought to have evolved to allow multicellular organisms to eliminate cells that are no longer required either to facilitate the development of the organism or because they are damaged or malfunctioning. A classic example of this process is the transformation that occurs during insect pupation that causes the metamorphosis of a worm-like insect larva into an adult insect often capable of flight. During this process, larval structures are dismantled which frees up their components for reuse in the production of new structures. In addition, in some instances apoptosis is induced by malfunctions in the cell cycle. This can include DNA damage or a variety of features of cells that are undergoing virus infection such as unscheduled DNA replication or RNA synthesis that is independent of the cell cycle. Therefore, apoptosis is also a major method by which organisms can limit and control viral infections.

Apoptosis is characterized by a specific series of events that are associated with cell death. These include loss of attachment to adjacent cells, cell shrinkage, nuclear fragmentation, chromatin condensation and DNA fragmentation and subsequent disintegration or blebbing of the cell into apoptotic bodies that are eliminated by phagocytosis. For a video of this process see [10]. Apoptosis is induced by processes associated with baculovirus DNA replication in *Spodoptera frugiperda* cells [11] and involves the activation of a series of proteases called caspases. There are two types of caspases involved in apoptosis, initiator and effector caspases. Initiator caspases activate proforms (inactive forms) of effector caspases (procaspases) by cleaving them. Effector caspases then cleave other cellular proteins resulting in apoptosis. Some effector caspases specifically target cytoskeletal proteins thereby causing the initial morphological changes including detachment and cell shrinkage. The activation of nucleases and the targeting of cell structural elements cause the subsequent fragmentation of the cell. Although it is thought that apoptosis evolved for the removal of cells that are no longer needed by an organism, it was adapted as a cellular defense mechanism for the elimination of cells infected by viruses. This

greatly reduces the ability of viruses to establish infections. In AcMNPV infections yield can be reduced up to 15,000 fold for viruses lacking the anti-apoptotic gene, p35 [12] [13]. In some aspects, the monitoring for virus infection is thought to be integrally involved with the monitoring of the cell cycle. When a cell is determined by checkpoint monitoring to have aberrant features such as DNA that is damaged beyond repair, the cell can be directed to undergo apoptosis, and thereby be eliminated. Apoptosis can be induced by DNA damage caused by ionizing radiation and by a variety of toxic chemicals. It is thought that the apparatus that monitors the cell cycle interprets viral DNA replication as aberrant or damaged DNA and induces the cell to undergo apoptosis. The most recent evidence suggests that events associated with DNA replication are required for the induction of apoptosis. In these experiments, it was found that by silencing genes required for DNA replication using RNAi, apoptosis could be significantly reduced. In contrast silencing genes specific to late gene expression did not cause apoptosis [11]. From this it was concluded that either the replication genes themselves, or DNA replication were responsible for the induction of apoptosis.

The study of apoptosis is a relatively new field and baculoviruses have played a major role in understanding this process. Baculoviruses became widely used as vectors for protein expression in the mid-1980s. Initially, the protocol involved the production of recombinants by homologous recombination at the polyhedrin locus. The polyhedrin gene was the selectable marker and recombination resulted in occlusion negative virions that could be identified by examination of the plaques that they formed in cultured cells. The first baculovirus apoptotic inhibitor was discovered when a laboratory identified an occlusion negative plaque that was not expressing the intended recombinant protein, but that had aberrant features, including a small plaque size. These investigators brought this to the attention of Lois Miller's laboratory that specialized in baculovirus research. The mutant virus was found to induce apoptosis and consequently had a small plaque size and reduced budded virus production. Investigation of the genome of the mutant virus led to the discovery of a baculovirus gene called p35, much of which was deleted in this aberrant virus. It was found that under normal conditions p35 was capable of blocking the apoptotic pathway in cells infected with the wt virus, but in this mutant it was inactivated [14].

A second fortuitous observation occurred when a laboratory was expressing one of the proteinases involved in apoptosis, caspase 1 (also called ICE) in the baculovirus system and found that it consistently co-purified with a contaminating protein. This contaminant was subsequently shown to be p35 and it was found to be a substrate for an effector caspase, caspase 1, but in the process of its cleavage it irreversibly binds to and inactivates caspase 1. Therefore, when caspase 1 was expressed in the baculovirus system, it bound to p35 that was also being expressed by the baculovirus. This led to understanding how p35 was able to block the apoptotic pathway [15] (Fig. 7.2). Subsequently p35 was found to block other categories of caspases in a similar manner - reviewed in [16]. Although baculoviruses encoding p35 would appear to benefit by its presence, its evolutionary

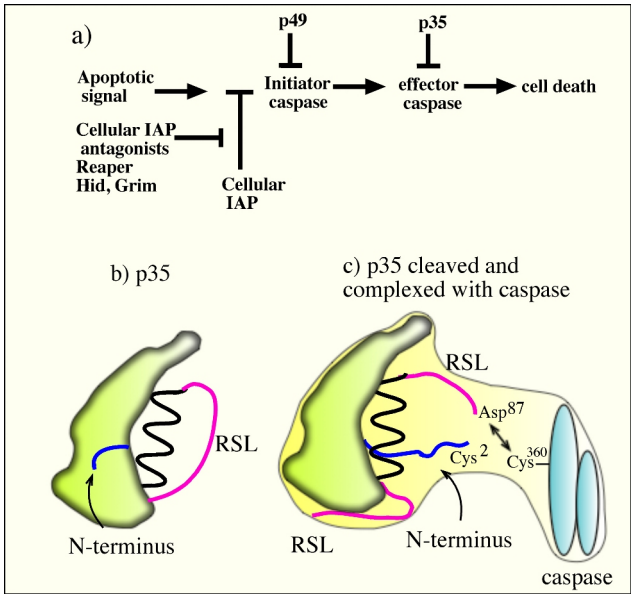

Fig. 7.2. Cell death regulation by p35. a) Baculovirus apoptotic inhibitors P49 and P35 prevent apoptosis by blocking caspase activity b) Intact P35 showing the reactive site loop (RSL) and N-terminus which is buried in the P35 structure. c) Cleavage and binding of p35 to caspase-8. P35 cleaved by caspase-8 at Asp^{87}. Asp^{87}forms a thioester bond with Caspase Cys^{360} which is in the proteolytic site thereby inactivating its enzymatic activity. Cleavage causes the N terminus of P35 to relocate near Asp^{87}. Cys^{2} near the N-terminus of P35 can also form a thioester with Asp^{87} forming a cyclic protein. This diagram is interpreted from [40] [41].

lineage is not clear. Closely related orthologs are only found in a few Group I baculoviruses closely related to AcMNPV. A homolog has also been reported in a GV of *Choristoneura occidentalis* (ChocGV) [17] and a variant (p49) is found in a Group II NPV (SpliNPV) [18]. In addition, a p35 ortholog was identified in an entomopox virus. The entompox virus p35 ortholog functions similarly to P35 in blocking effector caspases [19]. In contrast, P49 inhibits initiator caspases [20]. Despite its limited distribution to a few insect viruses, p35 is capable of blocking the apoptotic pathway in diverse organisms from invertebrates to mammals and has been a critical reagent in understanding the molecular interactions involved in the apoptotic pathway.

After the initial discovery of P35, to understand its distribution and diversity, its presence in other baculoviruses was investigated. This involved a granulovirus (CpGV) and an NPV, OpMNPV, that had been shown to have a deletion at the position where *p35* was predicted to be located [21]. Complementation was used to rescue an AcMNPV mutant deleted for *p35*. Unexpectedly, in both these viruses, a gene other than p35 was found to compensate for the lack of p35 [22, 23]. This gene was called inhibitor of apoptosis (*iap*). Subsequently, in contrast to the limited distribution of *p35*, *iap* orthologs were found in genomes of almost all baculovirus. Homologs are also found in other insect viruses such as entomopox and irridoviruses, and they are also widely distributed in eukaryotes, from yeast to mammals.

In addition to their widespread distribution throughout eukaryotes where it is often present in multiple copies, five lineages of iap genes (iap 1-5) have been identified in baculoviruses. Often an individual baculovirus will have representatives of several iap groups in their genomes, e.g., OpMNPV has representatives of iap -1, 2, 3, and 4 [24]. In addition, some viruses have two members of one of the lineages [25]. The distribution of iap lineages is as follows: iap-1 is only found in Group I NPVs, iap-2 is present in both Group I and II NPVs, iap-3 is found in Group I, II, GVs and hymenopteran NPVs, iap-4 is present only in a few genomes (3 Group I and 1 Group II NPVs as of 2007), and the iap-5 lineage appears to be confined to granulovirus genomes and is present in all but one of the GV genomes sequenced as of 2008. In the exception, AdorGV, two members of the iap-3 lineage are present [25]. Although the genomes of CpGV and OpMNPV were not completely sequenced when their iap genes were initially identified, both of these were later found to be members of the iap-3 lineage. Subsequently, iap-3 orthologs have also been shown to be anti-apoptotic in other baculoviruses [26, 27] (see below). Iap-3 genes are closely related to *iap* genes of insects. For example OpMNPV IAP-3 is 57% identical to IAP from *B. mori* indicating that the *iap* gene was likely captured by viruses on one or more occasions. In addition, iap from *S. frugiperda* has similar properties to IAP-3 in terms of its structure and function [28]. The role of other *iap* genes in baculovirus biology is not clear. In EppoMNPV, deletion of the *iap-1* gene, delayed the onset, but did not prevent apoptosis induced by actinomycin. However the *iap-2* ortholog from this virus was found to have anti apoptotic activity when expressed from a CMV promoter in *S. frugiperda* cells [29].

IAP sequences have a number of distinguishing domains. These include baculovirus IAP repeat (BIR) domains of about 70 amino acids. BIR domains are often present in multiple copies with two copies present in many baculovirus IAPs and up to three copies in some cellular IAPs. A zinc (RING) finger domain is also often present near the C-terminus of the protein. Such domains are often involved in protein-protein interactions. It has been found that the BIR domains of cellular IAPs block selected caspases. For example, in human X-linked iap (XIAP), the BIR2 domain binds to and inhibits caspase 3 and 7, whereas BIR3 inhibits caspase-9 - reviewed in [30]. Other IAPs have ubiquitin ligase activity associated with their RING domain and thereby target caspases for ubiquitination and subsequent degradation - reviewed in [31]. In *Drosophila* there are several proteins, e.g., HID, Grim and Reaper that block the activity of *Drosophila* IAPs and are called IAP antagonists (Fig. 7.3). These antagonists are necessary to allow for normal insect development that is based on the orderly removal of unneeded tissues via apoptosis. Therefore, they facilitate apoptosis by blocking proteins that would inhibit this process. Whereas, XIAP inhibits caspases, the most well-characterized baculovirus IAP, Op-IAP-3 is thought to act against the IAP antagonists, HID, Grim and Reaper. By inactivating the antagonists, the cellular IAPs are no longer bound and upon release can then block apoptosis (Fig. 7.3).

In summary, evidence suggests that homologs of the baculovirus IAP genes are capable of blocking apoptosis by several mechanisms including direct interference with caspases,

targeting caspases for degradation via ubiquitin ligase activity, and interference with antagonists of other proteins that block apoptosis. There is also another group of BIR-containing proteins that are involved in chromosome segregation and cytokinesis - reviewed in [32]. Unlike p35 that has a limited distribution and indeterminate origin, the baculovirus IAP genes have multiple homologs in their insect hosts that would have provided a source for the incorporation of an IAP gene on multiple occasions.

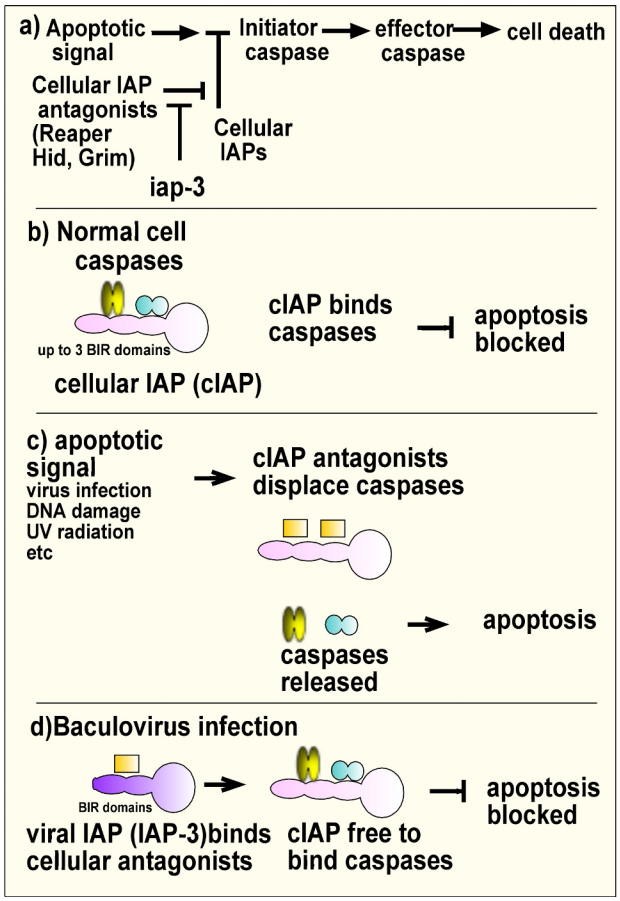

Fig 7.3. A possible mechanism for baculovirus IAP to block apoptosis. a) Schematic diagram of theory. b) Cellular IAP (cIAP) is related to baculovirus IAP and in the normal cells blocks apoptosis by sequestering caspases. c) Upon induction of apoptosis, cIAP antagonists displace caspases. This frees up the caspases to cause apoptosis. d) It is thought that baculovirus IAP blocks apoptosis by binding cIAP antagonists, thereby freeing up cIAP to bind caspases.

AcMNPV orf92 (p33), a link between apoptosis and the cell cycle?

Orthologs of Ac92 are present in all sequenced baculovirus genomes and it is an essential gene as viable recombinants deleted for this gene have not been isolated [33, 34]. Ac92 has been demonstrated to have sulfhydryl oxidase activity suggesting that it is involved in the formation of disulfide bonds [35]. It is also found associated with BV and ODV [33, 34] suggesting that it may be involved in the formation of disulfide bonds in the nuclei of infected cells. AcMNPV p33 also interacts with p53. P53 has been called 'the guardian of the genome' because of a number of roles it play in protecting cells from damage. Although normally inactive or expressed at low levels, it is induced by factors causing DNA damage, ribonucleotide depletion, and deregulated oncogene expression and can facilitate blocking the division of such cells. This interruption in the cell cycle allows time for the cell to repair the damaged DNA or it can lead to the induction of apoptosis and the destruction of the cell. Similar to the interaction with Rb, some viruses express proteins that inactivate p53 thereby they interfere both with its regulation of the cell cycle, and also prevent it from inducing apoptosis. Therefore it was of considerable interest when it was observed that Ac92 (p33) forms a stable complex with p53 [36]. When expressed by itself, p33 shows diffuse cytoplasmic staining and punctate staining of nuclei. However, when co-expressed with p53, it exclusively localizes to nuclei. Expression of human p53 in Sf cells causes apoptosis which can be blocked by co-expression of baculovirus anti-apoptotic suppressors p35 or OpIAP. However, co-expression of p53 with p33 elevated the induction of apoptosis about two fold. Orthologs of human P53 have been identified in insect genomes such as *Drosophila* [37]. Proteins with sulfhydryl oxidase activity have been implicated in the protection of cells from oxidative stress caused by apoptosis [38] [39]. The potential role of p33 in affecting the oxidation state of p53 and the effect this might have on the function of an insect p53 ortholog needs to be investigated.

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Chapter 8

Host resistance and susceptibility

Viruses are dependent on a variety of cellular functions to successfully infect an organism. For a productive infection, cellular structures and molecular pathways must be compatible with the virus for all the major events in virus replication including attachment, entry, uncoating, replication, assembly and exit. Insects, like many other organisms, have evolved methods to inhibit or block virus infections. Several methods by which organisms inhibit virus infection are summarized below. For a recent more detailed review of some of the topics in this chapter, see [1].

The insect immune system: hemocytes, melanization and encapsulation

Although lacking an adaptive immune system similar to vertebrates, insects have a variety of methods to resist infection by pathogens. Many of these defense mechanisms are similar to those of higher organisms and include physical barriers along with local and systemic responses. The latter include the production of antimicrobial peptides and highly specialized cells called hemocytes. Hemocytes are circulating cells found in the hemolymph of insects and other arthropods and are similar to neutrophils and carry out roles in immunity such as phagocytosis, encapsulation and melanization as a first line of defense after injury or invasion by microorganisms. Although not employed by mammals, melanization causes a blackening of a wound site, which results from the synthesis and deposition of melanin. It is an important defense against infections because it can encapsulate and isolate pathogens similar to the blood clotting system in vertebrates. In addition, the intermediates of the melanization reaction can involve the production of reactive oxygen species that can be directly toxic to pathogenic microbes. In arthropods, an inactive form of phenoloxidase (prophenoloxidase) is synthesized and secreted into the hemolymph. Melanization is regulated by a cascade of serine proteases that cleave and activate prophenoloxidase (to phenoloxidase) that is then able to catalyze the oxidation of phenols (e.g., tyrosine) to non aromatic ring compounds called quinones, which then polymerize and form melanin [2]. It has been suggested that phenol oxidase may have an antiviral effect in the plasma of insects in addition to its role in encapsulation [3]. The Toll pathway is another component of the innate immune response in many organisms. It acts by the recognition of structurally conserved molecules associated with pathogens such as viruses that are not present in the host organism. This recognition activates a cellular immune response. In *Drosophila*, melanization requires activation of the Toll pathway and is dependent on the removal of a serine protease inhibitor (Serpin27A), thereby allowing the cleavage of prophenoloxidase. The Toll pathway has been shown to be required for the efficient inhibition of Drosophila X virus. Inactivation of this system led to rapid death of infected insects and elevated virus titers [4].

Although most of the characterization of components of insect immune systems has been described from *Drosophila*, investigations have been conducted on baculovirus infection of Lepidoptera that suggest that they have similar defense systems. *Heliothis virescens* (tobacco bud worm) and *Helicoverpa zea* (corn earworm) are both members of the Noctuidae, but *H. zea* is 1,000-fold less susceptible to AcMNPV infection than *H. virescens*. Larvae of the two species are similar in their susceptibility to primary infection of the midgut and secondary infections of the tracheal epidermis. However, in *H. zea,* the foci infections in the tracheal epidermis become melanized and encapsulated and hemocytes appear to be resistant to infection and are capable of removing the virus from the hemolymph. Therefore, AcMNPV infection of *H. zea* appears to be able to activate host melanization and encapsulation responses that reduce viral titers in the hemolymph and inhibit the progression of the infection. In contrast, in *H. virescens* this pathway appears to be much less active [5].

The reaction of host cells to baculovirus infection: evidence from proteomics, microarrays, and expression analyses

AcMNPV was found to induce several host proteins during permissive infections of *Helicoverpa virescens* (Hv-AM1) cells [6]. These included DNA supercoiling factors that are involved in transcriptional regulation in other systems. Calriticulum was also up regulated. It is involved in maintaining homostasis of the endoplasmic reticulum and also can be involved as a chaperone in the folding of nascent glycoproteins. These would be important functions for the virus to maintain. Two heat shock proteins (HSPs) were also up regulated. They also serve as chaperones in protein folding. Similarly HSPs were also up regulated during AcMNPV infections of *S. frugiperda* cells and inhibition of these proteins significantly reduced the rate of DNA synthesis (20 fold reduction) and BV production (10 fold reduction) in infected cells [7]. Micro array analysis of BmNPV infections of *B. mori* cells identified elevated mRNA for transcription factors of the ETS family, a matrix metalloproteinase, a toll family gene, ATP binding cassette (ABC) transporter an alkaline nuclease, and several potential cell surface receptors [8]. Other investigations on the effects of BmNPV infection have identified certain mitogen activated protein kinases (MAPKs) that appear to be required for BmNPV gene expression. Inhibition of the expression of some these genes results in reduced virus production [9]. A comparison of cell lines derived from resistant and susceptible strains of *B. mori* found that two proteins, beta-N-acetylglucosaminidase 2 and aminoacylase were up regulated in the resistant cell line [10]. Beta-N-acetylglucosaminidase 2 is involved in processing carbohydrates of glycoproteins and it was suggested that it might be involved in resistance by altering the N-linked glycans of the viral GP64 envelope fusion protein and thereby reduce its ability to infect cells. Aminoacylase belongs to a group of enzymes that hydrolyze N-acetylated proteins. N-terminal acetylation of proteins is common and GP64 has been reported to be acylated [11]. Therefore the aminoacylase could also interfere with GP64 function [10]. Another study comparing resistance in B. mori identified eight genes that were up regulated in the midgut of resistant strains following BmNPV infection. These included genes encoding two antibacterial peptides, a serine protease (serpin) that might function in prophenol oxidase activation, cathepsin B, a cysteine proteinase, and actin A3 thought to be involved in the host immune response [12]. A comparison of gene expression of AcMNPV and BmNPV in resistant cell lines indicated that most of the AcMNPV genes were expressed in BmN cells, however their expression was delayed and very late gene expression was dramatically reduced. In contrast, in BmNPV infected *S. frugiperda* cells, the expression of almost all viral genes was severely restricted [13].

Other pathways that modulate virus infection

RNA interference. RNA interference (RNAi) has the potential to inhibit the infection of insects by some RNA viruses. In *Drosophila* evidence suggests that virus-specific dsRNA replication intermediates of RNA viruses are released upon cell lysis and taken up and processed by other cells thereby generating systemic virus-specific RNAi based interference [14]. However, some viruses can also interfere with this response. In one well-studied example, Flock house virus encodes a protein called B2 that suppresses RNAi by inhibiting the ability of Dicer to cleave long dsRNAs thereby preventing the generation of siRNAs. In addition, by binding to siRNAs, B2 prevents their incorporation into the RISC complex that is dependent on their incorporation to identify and cleave homologous RNA sequences thereby blocking the cleavage of target RNAs, reviewed in [15]. Although it was originally thought that flies were not able to generate a systemic RNAi response, it was recently shown that an uptake pathway for dsRNA exists in *Drosophila*. This pathway is essential for antiviral immunity in adult flies. Mutants defective for this system had up to a 100 fold increase in viral titers [14].

Two different components of RNA interference pathways have been demonstrated to suppress the activity of endogenous retroviruses in Drosophila. These include the PIWI RNAs (piRNA) in germline cells [16] and siRNAs in both germline and somatic cells [17] [18] (see Chapter 11). Although RNAi has been used to experimentally manipulate baculovirus infection of cells, AcMNPV replication does not appear to be affected by this system in permissive cells. However, this does not rule out that the host range of other baculovirus - insect combinations

can be affected by this system. In addition, the expression of miRNAs by large DNA viruses has been documented [19] and it remains to be determined if baculovirus employ such systems to regulate the expression of their host or their own genes.

Similar to other viruses, baculoviruses likely encode microRNAs [19] that are involved in the regulation of both host and viral genes. In one study, four miRNAs were predicted from analyses of the BmNPV genome [20]. The data suggested that they were targeted against 8 viral and 64 cellular genes. The viral targets included genes involved in replication, transcription e.g. dbp, DNA polymerase, and lef-8. Two different bro genes, chitinase, and several other genes were also identified. Some of the cellular targets involved host defense pathways that are likely involved in resisting the virus infection. These include prophenoloxidase which is integrally involved in the melanization response described above. Hemolin, another antiviral compound, also was predicted to be targeted. It is involved in pattern recognition, hemocyte aggregation, and phagocytosis. Several proteins involved in the pre-miRNA response and mRNA initiation were also identified.

Developmental resistance. It was also observed that *H. virescens* larvae demonstrate increasing resistance to fatal AcMNPV infections as they age. In one investigation it was found that the progression of infection is much slower in fifth compared to fourth instar larvae that had been orally infected. It was suggested that resistance was caused by different physiology of the midgut or tracheal cells. In particular, it was noted that midgut cells might undergo major changes since their composition is altered late in the fifth instar as the gut develops as part of metamorphosis to adult cell types. It was also suggested that midgut cells may be sloughed at a higher rate in later larval instars [21]. Similar patterns of resistance to baculovirus infection has been observed in *Lymantria dispar*. Newly molted 4th instar larvae showed lower LD_{50} titers compared to larvae infected at 2-3 days post molting. Also the 2-3-day post molt insects showed a higher number of foci of infection that had been melanized and a reduced number of infected haemocytes [22, 23].

Inactivation of superoxides, Ac31, superoxide dismutase (SOD). Insect hemocytes are phagocytic cells (see above) and can destroy invading pathogens by the production of reactive oxygen such as superoxide [24]. SOD can inactivate superoxide by converting it to hydrogen peroxide, which is also toxic, but can itself be inactivated with catalase to yield water and O_2. Many baculoviruses may infect hemocytes and in this manner can spread an infection throughout an insect. Orthologs of SOD are found in the genomes of almost all lepidopteran baculovirus genomes. Ac31 (SOD) is closely related to SOD from a variety of insects including *B. mori* (E = 8E-49). The expression of viral SOD might mitigate the effects of superoxide production by hemocytes. However, AcMNPV deleted for *sod* replicated normally in cultured cells and insect larvae. The *sod*-deleted viruses showed no reduction in replication when grown in the presence of paraquat, a superoxide anion inducer [25]. Whereas, these data suggest that SOD of baculoviruses may be involved in some other role in virus biology, it could also indicate the conditions used to investigate its activity were not sensitive enough to determine its role. In contrast to AcMNPV, in BmNPV it was found that *sod* (Bm23) was essential for replication in BmN cells [26].

Host resistance to baculovirus infection in the midgut

The peritrophic matrix (PM). In addition to protection from external invasion by pathogens that is provided by the chitin-containing exoskeleton, an example of another physical barrier unique to insects that might influence resistance to viral infection is the PM. The ability of a virus to access the midgut epithelium would be one of the first problems confronting a virus when it is initiating infection. In some insects that are resistant to infection it was found that differing features of the PM can influence the susceptibility of an insect to viral infection. In *Anticarsia gemmatalis*, it was observed that insects that were more resistant to infection, had a relatively thicker PM than more susceptible insects [27].

Midgut interactions. It has also been demonstrated that the oral infectivity of a virus could be influenced by the ability of the ODV to bind to the midgut of insects. It was observed that compared to SfMNPV, AcMNPV ODV had a reduced affinity for midgut cells of *S. frugiperda* larvae. Evidence was also presented suggesting that SfMNPV might bind to a different receptor(s)

on columnar epithelial cells and this could contribute to the efficiency of its ability to initiate infection [28].

Other factors influencing Baculovirus host range

A variety of phenomena that govern the selective infectivity of baculoviruses have been covered previously and there is little to add to their review [29]. However, there have been recent contributions to understanding specific genes that have been observed to affect host range in insects. Most investigations on the molecular basis or host range specificity of baculoviruses have been done using BmNPV, AcMNPV, and LdMNPV. All of these viruses can be grown in cell culture and BmNPV and AcMNPV are closely related with homologous orfs showing ~90% nt and ~93% aa sequence identity [30]. In contrast, LdMNPV is a member of the Group II baculoviruses and its orfs show about 41% aa identity with their AcMNPV homologs [31]. Despite the similarity of AcMNPV and BmNPV, AcMNPV infects a much more diverse set of insects and insect cell lines than BmNPV [32]. Whereas some of the limits on host range that were initially observed in cultured cells extend through to infection of the host insects, other host range effects are limited to a cell line and are not as restrictive in other cell lines from the same insects or in insect larvae of the species from which the cell lines were derived. This section will focus on investigations of host range in AcMNPV and BmNPV.

Investigations on BmNPV and AcMNPV host range in *B. mori* and *S. frugiperda* cells

The baculoviruses of BmNPV and AcMNPV are closely related showing on average orf amino acid sequence identities of about 93% [30]. Despite their similarity they differ significantly in their infectivity spectrum. For example, BmNPV replicates in *B. mori* (BmN) cells, but not *S. frugiperda* (Sf) cells. Conversely, AcMNPV replicates in Sf, but not BmN cells. Although the two viruses do not appear to productively infect the heterologous cell line, their patterns of gene expression differ in the nonpermissive cell lines. For example, almost all of the AcMNPV genes were found to be expressed in both BmN and Sf9 cells, although peak levels were delayed by about 12 hr in the nonpermissive BmN cells and polyhedrin and p10 expression were substantially reduced. In contrast, although almost all of the BmNPV genes were expressed in BmN cells, their expression in Sf9 cells was greatly reduced [13]. Several different laboratories have investigated the factors responsible for the inability of these viruses to replicate in the heterologous cells. Some of these studies are summarized below.

Implication of DNA helicase in specifying host range in BmNPV and AcMNPV. By characterizing mixed infections of BmNPV and AcMNPV, a variant of BmNPV was isolated that was able to replicate in both BmN and Sf-21 cells. A 572 bp fragment of the BmNPV DNA helicase gene was found to be responsible for this altered host range [33]. Further characterization of this region identified a single amino acid in the helicase orf (Asn564Ser) responsible for this change [34]. Using a similar approach, other investigators found that altering three closely spaced amino acids in the AcMNPV helicase gene with the amino acids from BmNPV located at these positions, i.e., Val556Leu, Ser564Asn, Phe577Leu, allowed AcMNPV to replicate in *B. mori* cells [35]. When AcMNPV mutants selected for their ability to replicate in BmN cells were passed through *B. mori* larvae, two amino acids changes (Ser564Asn, Phe577Leu) were found to be required to cause death of the larvae [36]. It is unclear what role helicase plays in governing the inability of the virus to replicate in the heterologous cell lines. However, co-infection of BmN cells with wt AcMNPV and wt BmNPV causes premature cessation of both viral and host protein synthesis, although viral transcription appears to be normal. This effect is not observed when BmN cells are infected with both the recombinant AcMNPV and wt BmNPV. It has been suggested that since these few changes in the helicase gene have such a major effect on infection in BmN cells, that the wt AcMNPV helicase gene may be toxic to the cell by eliciting an antiviral defense mechanism by interacting with a host cell protein or nucleic acid or by interfering with the translational apparatus [37].

BmNPV may be infectious for Sf-9 and other cells, but the level is low. In contrast to the

reports described above, other laboratories using viral constructs expressing reporter genes have detected replication of BmNPV and AcMNPV in heterologous cell lines. In one study, a BmNPV construct expressing the LacZ gene under the polyhedrin promoter in Sf-9, Sf-21 and Hi-5 cells was examined. Compared with BmNPV infection in Bm5 cells, the BmNPV infections in the other cell lines were delayed with DNA replication detectable 3 to 5 days after being observed in BmN cells. In addition, the viral titers were much lower varying from 0.7 to 7% (10^6 to 10^7 pfu/ml) the level in BmN cells [38]. Since this report utilized LacZ expression to trace the virus, it may be more sensitive than previous studies that utilized the visualization of polyhedron production to monitor viral replication. Indeed, these authors detected either very few or no polyhedra in the heterologous infections using a wt BmNPV with an intact polyhedrin gene. In another investigation, using an AcMNPV construct that expressed the firefly luciferase gene under the *Drosophila* heat shock promoter, luciferase activity was detected in both virus infected larvae and in larvae of the next generation [39]. Therefore both these studies indicate that viral replication is occurring in the heterologous cell lines. Another report described AcMNPV DNA replication in BmN cells, but budded virions were not produced. In contrast, BmNPV DNA replication and BV production only occurred in Sf cells when they were superinfected with AcMNPV [40].

Implication of a host factor in specifying host range in BmNPV and AcMNPV [41]. In studies conducted by the intrahemocoelic injection of AcMNPV into 31 different strains of *B. mori* larvae, 14 permissive insect strains were identified. A series of genetic crosses implicated a dominant host gene or set of linked genes that prevented AcMNPV infection in the resistant insects, but that are not present or do not interact with the virus in a negative manner in the susceptible insects.

gp64: an AcMNPV and BmNPV host range determinant. The one study that is difficult to reconcile with the investigations described above examined the replication of BmNPV in Sf-9 and Tn-5 (Hi-5) cells. Their investigations suggested that the barrier to infection was caused by the inability of BmNPV to be translocated to the nuclei of the Sf-9 or Tn-5 cells. It was found that BmNPV constructs that lacked the BmNPV gp64 envelope fusion protein gene, but contained the AcMNPV gp64 gene, were able to be translocated to nuclei. Although this process resulted in a productive infection in Hi-5 cells, replication in Sf-9 cells was still compromised. There are 22 codons that are different between these two genes. There is also the potential that the BmNPV gp64 orf may encode 19 additional amino acids at the N-terminus in transcripts initiated from late promoter elements [32]. This study indicated that abortive replication was caused by the inability of BmNPV gp64 to facilitate fusion with the endosomal membrane. This would suggest that BmNPV GP64 has a major difference in biological properties compared to AcMNPV GP64, which has been demonstrated to allow entry into a wide array of vertebrate cells, e.g., [42]. Recent evidence suggests that a single amino is the host range determinant in AcMNPV GP64. It is HIS155 and the equivalent amino acid in BmNPV is TYR153. Changing AcMNPV gp64 to HIS155TYR and that of BmNPV to TYR153HIS, decreased and increased the replication and cell spread of the respective viruses [43].

One possible difference between GP64 expression in the two viruses is the observation that, in contrast to the AcMNPV gp64 gene, which is shut off late in infection of Sf9 cells, in BmNPV infected BmN cells, *gp64* is not shut off late in infection [44].

Host cell-specific factor-1 (*hcf-1*, Ac70): AcMNPV specificity for *T. ni* cells. Homologs of hcf-1 (Ac70) are present in only three other baculoviruses; two are close relatives of AcMNPV and their HCF-1 orfs are 99% (PlxyNPV) and 84% (RoMNPV) identical to that of AcMNPV, whereas the homolog in ClbiNPV is more distantly related (21% identical). HCF-1 was found to be required for transient expression of a late promoter-reporter gene by a late expression factor library in Tn368 cells, but not SF-21 cells [45]. AcMNPV with null mutations of *hcf-1* appeared to replicate normally in both Sf-21 cells and *S. frugiperda* larvae. However, in Tn-368 cells replication was impaired, including defects in DNA replication, late gene transcription, and virus production. This was reflected in the arrest of both host and viral protein synthesis. Such severe effects were not observed in another *T. ni* cell line. In *T. ni* larvae the oral infectivity of the null mutant was relatively

normal, although the insects died more slowly than when infected with wt [46]. This would suggest that HCF-1 is required for the productive infection of some, but not all cell types in *T. ni* larvae. Therefore, whereas AcMNPV deleted for *hcf*-1 was unable to replicate in cells similar to Tn-368, it can replicate in other types of *T. ni* cells thereby allowing infection of larvae that contain a variety of different cell types.

Host range factor-1 (*hrf-1*): An LdMNPV gene that allows AcMNPV to replicate in *L. dispar* cells and insects. The Ld652Y cell line is semi-permissive for AcMNPV replication and all categories of viral genes are transcribed, but both viral and host mRNA translation is blocked late in infection [47]. Cotransfection of LdMNPV and AcMNPV DNA into Ld652Y cells results in the production of budded virus that can replicate in Sf cells, suggesting that AcMNPV had been altered such that it could replicate in Ld652Y cells. To determine the LdMNPV gene responsible for this change, AcMNPV DNA was co-transfected with cloned segments of the LdMNPV genome and an LdMNPV gene was identified, *hrf-1*, that permitted AcMNPV to replicate in Ld652Y cells [48]. This recombinant AcMNPV was also able to infect *L. dispar* larvae in concentrations similar to wt AcMNPV in permissive insects [49]. It was found that wt AcMNPV infection of *L. dispar* cells resulted in a shutdown of protein synthesis late in the infection [50]. This protein synthesis inhibition was at the level of mRNA translation and could be rescued with tRNA from uninfected cells. This suggested that infection of *L. dispar* cells with wt AcMNPV results in the depletion or blockage of tRNA synthesis [51]. It was also found that AcMNPV, lacking the apoptotic inhibitor p35 did not cause translational arrest suggesting that the inhibition of apoptosis, which normally prevents cell death, in this case induces translational arrest [52]. It was found that HRF-1 facilitated the replication of two other viruses in *L. dispar* cells [53].

Apoptosis and the specificity of baculovirus infections. Insect cells have different abilities to detect the presence of a virus infection and initiate an apoptotic program. In some cells virus infection will induce apoptosis, whereas in others, virus replication is unaffected. In addition, baculoviruses are able to interfere with apoptosis by the expression of apoptotic inhibitors. This has led to advances in understanding of apoptotic pathways and to the identification of baculovirus proteins that can block this process. It has also led to an understanding of the role apoptosis can play in determining the host range of a virus. An overview of apoptosis with relation to baculoviruses is included in Chapter 7.

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Chapter 9

Baculoviruses as insecticides: Three examples

One of the earliest references for using natural pathogens for insect control was reported by V. Audouin in 1839, who tells of a sericulturist who emptied fungus-contaminated silkworm rearing trays out a window onto trees infested with defoliating insect larvae. Within a few days all of the defoliating insects had died of the fungus. More explicit suggestions for the microbial control of insects were made by J.L. LeConti in 1874 in an address to the AAAS, in which he recommended the study of epidemic diseases of insects and advocated their use to control insects. At the same time Louis Pasteur who had spent considerable effort investigating a microsporidian parasite of silkworms recommended the use of this pathogen against an insect pest of grapes (described in [1]).

In ecosystems, baculoviruses often play a major role in the suppression of a variety of different types of insects. For example, the virus of the gypsy moth, *Lymantria dispar,* is considered to be the major natural regulator of dense populations of this moth [2]. Likewise, baculoviruses of the Douglas fir tussock moth, *Orgyia pseudotsugata*, are also major factors in the control of this insect [3]. They also are found to naturally control pest insects of cultivated crops. For example, they were found to be major contaminants of cabbage purchased from supermarkets in the Washington, D.C. area and it was estimated that an average serving of cole slaw would contain over 100 million occlusion bodies [4]. Once the role these viruses played in the controlling natural insect populations was understood, they were considered for a variety of insect control programs particularly of forest pests [5].

There have been numerous reviews on the development of baculoviruses to control insects, e.g., [6-8], and despite this widespread interest and intrinsic attractiveness of their use, the acceptance and use of viruses for insect control has been limited. This can be attributed to their slow speed of kill, their limited host range such that one preparation can only be used on a few insects, and to a certain degree to the complexity of producing standardized viral preparations. The slow speed of kill may be of particular advantage to the virus, because it results in greatly increased viral yields. However, delays in the death of the host result in more vegetation being consumed by the infected insect. A variety of recombinant viruses have been investigated that have been designed to enhance the efficacy of the virus by reducing the time it takes to kill target insects or causing cessation of feeding. These recombinants express insect specific toxin, insect hormones or enzymes, or are deleted for the EGT gene. Contributing factors to their limited use for biocontrol is that production of viral insecticides is labor intensive and consequently their use has been limited to high value crops particularly those that have become resistant to chemical insecticides or to crops in countries with access to relatively inexpensive labor. An exception appears to be their use against forest insects in North America, however relative to the size of forested areas, these applications are also limited. In addition, the use of *Bacillus thuringiensis* preparations is highly competitive compared to baculoviruses because of the simplicity of their growth and formulation. In this chapter, I will review three instances where viruses have been relatively extensively applied in the field.

An NPV of the velvet bean caterpillar, *Anticarsia gemmatalis*: Application in Brazil.

The most successful program employing baculoviruses for insect control has been developed in Brazil and to a lesser extent in Paraguay and involves a virus that infects the velvet bean caterpillar (*Anticarsia gemmatalis*), a pest of soybeans [9-11]. Virus preparations are applied at 1.5×10^{11} occlusion bodies per hectare (about 20 g or 50 larval equivalents). This program was initiated in the early 1980s and by 2005, the area treated had expanded to over two million ha ([7] and references therein). Initially laboratory production was not found to be economically viable and consequently virus production was carried out in farmers' fields.

Plots of soybeans that were naturally infested with *A. gemmatalis* were sprayed with virus and then the dead larvae were collected. Individuals were able to collect about 1.8 kg of larvae/day at a cost in the mid 1990s of about $15. Production varied in the 1990s from enough virus to treat 650,000 to 1.7 million ha/year. Since 1999, the production of virus has not been sufficient to meet the demand. Consequently, to increase virus production under more defined conditions a laboratory for the large scale production of AgMNPV was opened in 2004 with the intention of being capable of infecting 600,000 larvae per day. This would have the potential to yield enough virus to treat up to 1.5 million ha/year. Another facility was opened for the improvement of virus production and for the training of individuals for virus production. It has the capacity to infect up to 30,000 larvae per day ([7] and references therein). The infected larvae are processed into a wettable powder that involves milling the infected larvae and formulating them into a mixture that contains kaolin. Kaolin is an aluminosilicate compound first discovered in Kao-lin, China that is commonly used as an inert carrier or filler. Some of the reasons for the feasibility of this viral control program are caused by specific features of AgMNPV. First, the virus is highly virulent for *A. gemmatalis* and usually only needs to be applied one time. In contrast, chemical insecticides often need to be applied twice. Furthermore, AgMNPV lacks the chitinase and cathepsin genes and consequently the insects die without 'melting' and the dead insects can be more readily collected and processed than if they had disintegrated [12] (see Chapter 3). Another factor contributing to the success of the program is that soybean plants can endure significant defoliation without a reduction in yield. The virus can also be spread by insect predators and can survive passage through the digestive tract of beetles and hemiptera [11]. Overall, the use of the viruses is 20-30% less expensive than chemical insecticides and it has been estimated that the use of up to 17 million liters of chemical insecticides has been eliminated since the beginning of the program. Limitations have included the reluctance of farmers to monitor their fields to determine the optimal timing for virus application and its use in regions that have low mean temperatures, which lengthens the time required to kill the insects. Extended periods of drought also adversely affect the efficacy of the virus preparation ([7] and references therein).

A granulovirus of the codling moth, *Cydia pomonella*: Application in North America and Europe.

Whereas the use of AgMNPV has been limited predominantly to one major area in Brazil, a granulovirus of the codling moth *Cydia pomonella* (CpGV) has been used in a number of countries in North America and Europe for the control of the insect on pear and apple crops. CpGV was originally isolated from *C. pomonella* in Mexico in 1963 [13]. Because of the development of resistance of codling moth to several chemical insecticides and for a variety of other safety and environmental reasons, the use of CpGV has increased in Europe and North America since 2000 and the virus is used on a hundred thousand or more hectares on these continents. Currently commercial preparations of the virus are available from several different companies and include preparations called Cyd-X and VirosoftCP4 in North American and in Europe, include Carpovirusine™, (France), Madex™ and Granupom™ (Switzerland), Granusal™ (Germany), and Virin-CyAP, (Russia). The virus is highly virulent for codling moth with LD50's as low as 1.2-5 occlusion bodies per insect. The codling moth lays eggs on fruit trees and after hatching, the larvae browse on leaves before entering fruit. They need to feed inside fruit for normal development and this can result in severe damage. Depending on the climate, there can be from one to three generations per season and to ensure exposure during the brief window from hatching to entry into fruit requires the application of CpGV at least at weekly intervals up to six times in a season.

Recently resistance to the virus has been described in Europe and these insects can tolerate CpGV over 1,000 times higher than previously observed. In laboratory experiments, it was determined that a gene conferring resistance is located on the male (Z) chromosome and it was found that females with a single Z chromosome could be selected that were almost 100,000 time less susceptible to the CpGV infection [14]. Because of the complexity of baculovirus replication it was often assumed that it would be challenging for an insect to develop resistance. However, these results clearly indicate that the alteration of a single or limited number of linked genes can severely compromise the infectivity of these viruses.

Although the mechanism of CpGV resistance is not clear, its evolution emphasizes how dependent baculoviruses are on their hosts for carrying out their replication cycle and a change in a single receptor or other protein, such as would be required for DNA replication, can interfere with virus infectivity.

An NPV of the cotton bollworm, *Helicoverpa armigera*: Application in China

The cotton bollworm, *Helicoverpa armigera,* is a major pest of cotton and with the intensive use of chemical insecticides it has developed resistance in many parts of the world. One approach to counteract this resistance has been the use of baculoviruses for control of this insect. In China, HearNPV has been produced for use against the cotton bollworm. In the most recent available data (from 2005), about 1,600 tons of infected insects were processed by 12 different producers [15]. The insects were grown on artificial diet composed of mainly corn and wheat and the infected larvae were processed after removal of lipids into wettable powders or emulsions. Treatment involves spraying fields 3 to 5 times per growing season to control two generations of the cotton bollworm. It was estimated that the virus preparation was used on 200,000 to 300,000 hectares of cotton in 2005. In India, it has been reported that insects are collected by shaking larvae off pea plants onto blankets. HearNPV is then produced by feeding the larvae virus contaminated chickpea seeds [7].

A recombinant HearNPV is being evaluated in China that expresses a gene encoding an insect-specific toxin (AaIT) from a scorpion found in the Middle East and Africa called *Androctonus australis*. The use of this recombinant baculovirus is limited to experimental plots of about 2 hectares. In this construct the AaIT gene is inserted at the EGT locus. This causes problems with the production of the recombinant virus in infected insect larvae. Since the toxin is active against larvae and deletion of the EGT gene reduces the time it takes the virus to kill the insect, the levels of production are significantly affected and under optimal conditions virus yield is less than 50% of wt. However, the yield from cotton plants treated with this virus is about 25% higher than from plants treated with wt virus. Consequently, this recombinant has significant advantages over the wt virus [15].

A variety of other viruses that are being produced in China range from 120 tons of AcMNPV to less than 50 tons of a several other viruses in 2005. These were used to control a variety of insects mostly on vegetables and tea. The data described above is from [15] and Xiu-lian Sun (pers. Comm.).

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Chapter 10

The evolution of high levels of baculovirus gene expression

Some History

I participated in a symposium on microbial pesticides organized by Lois Miller as part of a regional American Society for Microbiology meeting in Moscow, Idaho in mid-June 1983. At that meeting she showed me some pictures of a baculovirus expression system - blue β-galactosidase plaques on *S. frugiperda* cells – developed in her laboratory by an undergraduate student, Greg Pennock. She indicated that she was using them as cover pictures for an issue of Cell to which she had submitted a manuscript describing this work. Later that summer, at the most riveting American Society for Virology workshop that I ever attended, she described this system as did Gale Smith who showed baculovirus expression of an interferon. Gale Smith and Max Summers published their report later in the year [1]. Lois Miller's report was rejected by Cell thereby delaying publication until 1984 [2] and giving Max Summers and Gale Smith clear precedence with regard to this technology. In addition, their production of a practical manual detailing the use of the system [3] and distribution of its major components greatly facilitated its widespread application before it was commercially available. The development of this technology gave birth to a new era in baculovirology based on targeted gene recombination and has resulted directly or indirectly in much of the progress in understanding the biology of these viruses along with advances in many other biological systems dependent on protein expression technology. The variations and modifications of this system are not within the scope of this book and have been ably covered in a several recent reviews, e.g., see [4]. However, I will give a brief overview of the combination of biological features of insects and baculoviruses that have contributed to the evolution of a system of such utility for biotechnology. These are summarized in Table 10.1.

Table 10.1 Major Factors influencing high levels of baculovirus very late gene transcription

	Factor	Result
1	Ability of virus to cause systemic infection	Allows virus to exploit insect synthetic systems, e.g., the fat body
2	Shut off of early baculovirus and host gene transcription	Makes host cell biosynthetic systems available for baculovirus gene expression
3	Shut off of baculovirus late gene expression	Makes baculovirus RNA polymerase available for very late gene expression
4	DNA replication and a high concentration of unpackaged viral DNA	High copy numbers of very late genes are accessible for transcription
5	Efficiency of the baculovirus RNA polymerase	Facilitates high level mRNA production and RNA capping
6	The biosynthetic capacity of insect cells	Allows high levels of very late gene expression

Initiating infection: environmental stability and the insect midgut

Insect species are the predominant terrestrial eukaryotic animals both in terms of species numbers and also in their contribution to the terrestrial biomass. After Coleoptera, members of the family Lepidoptera are some of the most numerous in terms of species numbers. Members of this family are phytophagous and in temperate climates and in many tropical areas where there are distinct wet and dry seasons, insect populations are transitory and expand dramatically during warm, moist periods, and then collapse with the onset of colder temperatures or drought when food sources are reduced or eliminated. In addition, even during seasons optimal for insect growth and reproduction, their populations are normally limited by predators, pathogens, normal temperature cycles, and food sources. However, under certain circumstances, insect populations can expand dramatically when a combination of conditions greatly increases their food supply, facilitates high levels of reproduction, or eliminates predators and pathogens. These cycles of population expansion are well-documented for forest insects and may be separated by long periods of time. The brief life cycle of most Lepidoptera, that often is limited to a few weeks, allows them to rapidly respond to such environmental conditions. For example, typical lepidopteran larvae, e.g., *M. sexta*, are capable of exponential growth that can be particularly dramatic in the last larval instar, during which a 12 fold increase in mass (from 1g to 12 g) in as few as 4 days has been documented [5]. However, considering the small size of their eggs an overall increase in mass of several thousand fold occurs over the 3 weeks of their development. The capacity of Lepidoptera to both ingest and assimilate large amounts of food combined with their prolific reproductive abilities are correlated with the devastating losses that they can inflict on both native and cultivated vegetation. One of the purposes of these levels of synthesis is for the production of lipids and proteins that are involved in egg production in females and also in the major transformations that occur during metamorphosis [6]. In addition, adult Lepidoptera lack mandibles and either shift to nectar feeding via an extendable proboscis, or do not feed at all thereby necessitating the storage of energy during the larval stage. Consequently, because of their ability to proliferate to high population densities and their metabolic potential, Lepidoptera provided an attractive target for viral parasitization.

Several major problems had to be solved to allow for baculoviruses to successfully infect Lepidoptera. First, they had to evolve a method of infection. All baculoviruses appear to normally infect insects via the digestive tract as contaminants of the food supply. The insect midgut provided viral access to cells that lacked a resistant chitinous surface. Second, they had to develop a method of persistence so that they could survive the interludes between the population cycles of their hosts. Third, they had to confront the environment of the insect midgut that attains some of the highest pH levels recorded in nature. At the entry and exit of the midgut, the pH is commonly near 7.0, however in the central region, levels of 10.0 to as high as 12.0 have been recorded [7] (see Chapter 3, Fig. 3.3). The problems of environmental stability, and the high pH of the insect midgut, have been solved by a several apparently unrelated viruses in a similar manner. This solution involves incorporation of virions into an alkali-soluble occlusion body that provides stability outside the host insect, allowing them to persist in the environment between insect populations. It also provides for the release of the virions, when an environment conducive to infection is encountered. Few if any areas of such high pH are present in nature, except for the lepidopteran midgut. The combination of occlusion with alkali solubility has been so successful that it has apparently evolved independently on at least three occasions; with the baculoviruses, the cypoviruses, and the entomopox viruses. All are insect viruses that infect their hosts via the midgut, but are from completely different viral families. In all cases, the consequence of occlusion is the fact that all these different viruses have developed the ability to express one or two proteins at very high levels. These form the occlusion body matrices.

The infection of insects requires that the occluded virions be liberated from the protein matrix and this is accomplished by the alkaline conditions encountered in the midgut. Surviving and initiating infection under these conditions is apparently such a highly specialized task that many of the proteins that are involved in this process are conserved throughout not only the Baculoviridae, but also in a related group of nonoccluded viruses, the nudiviruses. Many of

what are considered the more primitive baculoviruses appear to limit their infection to the gut cells. However, when limited to this tissue, their reproductive potential is greatly diminished, because they do not have access to systemic infection, in particular, to fat body cells that comprise the major metabolic organ of lepidopteran larvae.

Evolution of a biphasic replication cycle that allowed exploitation of the biosynthetic capacity of insect systems

The ability of baculoviruses to exploit the power of the lepidopteran metabolic system was likely facilitated by their ability to move from the midgut to cause systemic infections. This required the production of a viral type that could initiate infection in a completely different environment: one in which the pH was near neutral and that lacked the high concentrations of enzymes that the occluded virions had evolved to survive. Consequently, baculoviruses appear to have incorporated a more conventional envelope fusion protein, called the F protein, which is similar to the pH-dependent fusion proteins of many viruses. This protein is expressed by the virus, modifies the cytoplasmic membrane and is obtained by the virus when it buds out of the infected cells. It allows the virus to infect a wide variety of cells throughout the insect and thereby provides access to the prodigious metabolic potential of the insect larvae. Subsequently another fusion protein, called GP64, displaced the function of the F protein and is the fusion protein of one branch of the baculoviruses that is the focus of much of the research in biotechnology.

Optimizing the cellular environment: The viral RNA polymerase and the shut down of most viral and host genes late in infection

Upon infecting cells, baculoviruses appear to be able to focus transcription on their own genes probably by the expression of the powerful transactivator IE1 along with the presence of enhancer sequences on the viral genome (see Chapter 5). In addition, a major innovation was their incorporation of an RNA polymerase. The viral RNA polymerase allows exploitation of the insect synthetic machinery independent of the constraints of host cell transcription.

In addition to exploiting the synthetic capacity of insects that became accessible by the evolution of systemic infections, several other factors contributed to the evolution of high-level baculovirus gene expression. Identification of one of these factors was based on the observation that most baculovirus genes are shutdown after they have been expressed. An example is the gp64 gene of OpMNPV that has both early and late promoter elements [8]. The early promoter is activated shortly after infection (6 hr pi in *L. dispar* cells) and continues until 36 hr after which it declines. Activation of the late promoters occurs between 24 and 36 hr p.i. and then also declines. The vp39 gene, which only has a late promoter element, showed similar kinetics [9]. In contrast the polyhedrin and p10 gene transcripts are present very late in infection (60 hr p.i.) [10, 11]. In addition, it has been demonstrated that AcMNPV infections of *S. frugiperda* cells leads to a reduction in levels of almost all host cell mRNAs by 24 hr p.i. [12]. The shutoff of most host genes would likely make both the cellular transcriptional and translational apparatus available so that it can be focused on the expression of viral genes. Similarly the shutoff of viral late genes likely frees up the viral RNA polymerase so that it can focus on the transcription of polyhedrin and p10. It is not clear what governs the shut off of viral and host genes. The viral transactivator, IE1, along with *hr* enhancer sequences, may divert early transcription from host cell genes to viral genes. This could lead to the shut down of most host genes. The expression of baculovirus late genes occurs after DNA replication is initiated. This could be facilitated by the presence of newly replicated (naked) DNA, or by the presence of the unligated junctions of Okazaki fragments that may serve as transient enhancers of late transcription by acting as loading sites for late gene activators that track along the DNA until late promoters are recognized [13] (see Chapter 6). Once DNA replication is completed, these loading sites for late gene activators are no longer available and this may be reflected in the turn off of late genes.

Very late gene (p10 and polyhedrin) activation and transcription

If viral late genes are shutdown by the accumulation of DNA binding proteins, very late gene transcription may be induced by the expression of a transcriptional activator (Fig. 6.4). As described in Chapter 6, VLF-1 is one of the few genes that has been implicated in the selective up regulation of very late genes and has been shown to interact with the burst sequence of p10 and polyhedrin regulatory regions in gel shift assays [14]. LEF-2 [15, 16] and protein kinase 1 (Ac10) [16-19] may also be involved in this process. In addition to focusing very late transcription on polyhedrin and p10, the high levels of transcription could be influenced by the efficiency of the baculovirus RNA polymerase in transcribing these two genes and also the ability of LEF4 to cap the very late transcripts so that they can be transported and translated efficiently.

A role for gene copy number and nonencapsidated viral DNA

Another major contributing factor to high levels of very late gene expression is likely to be a high copy number of polyhedrin and p10 genes. Baculovirus very late gene expression is fundamentally concerned with the occlusion of nucleocapsids. However, in order for very late genes to be transcribed, it is likely that a significant portion of the DNA synthesized during infection is never packaged into nucleocapsids and is lost at the end of the infection cycle. It is critical that this DNA is not incorporated into nucleocapsids, because once incorporated, it would not be accessible to the RNA polymerase. Indeed, it has been observed that, whereas 100% of BV DNA is resistant to DNAse, up to 70% of viral DNA present in extracts of cells late in infection was sensitive to DNAse, suggesting that it was not packaged and is therefore accessible to digestion [20]. Furthermore, it has been observed that, whereas a portion of the DNA in infected cells can be converted to unit length DNA, most of the DNA appears to be present in complex, possibly branched structures [21]. This has led to the suggestion that there are two categories of baculovirus DNA (see Chapter 5); DNA destined to be incorporated into nucleocapsids, and DNA that is never packaged because it is required for very late transcription. The synthesis of DNA incorporated into nucleocapsids is likely coordinated with its packaging into virions, whereas the extra-viral DNA is synthesized independent of packaging and undergoes extensive recombination because it is not protected from this process by incorporation into virions. The likely complex structure of the unpackaged DNA would probably not interfere with high levels of very late transcription.

Baculovirus gene expression and biotechnology

As described above, the biosynthetic capacity of baculoviruses is derived from the intersection of their infections with the growth of their hosts. This results in the diversion of the cellular macromolecular material and energetic capacity to viral rather than insect growth. The ability to recreate this environment in the laboratory was dependent upon the isolation of insect cell lines permissive for baculovirus infection, and the identification of growth conditions that allow for optimal viral replication in these cells. The isolation and development of a variety of insect cell lines to be both permissive for baculovirus replication and to have the capacity to undergo exponential growth has allowed for the exploitation of the combination of both the insect synthetic capacity and the virus in the form of the baculovirus expression system.

Summary and conclusions

It is likely that several factors have combined to make baculoviruses highly efficient in gene expression. This includes gene amplification, the shutoff of most other genes very late in infection, the specific activation of very late genes, efficient gene transcription, and access to the protein synthetic machinery of the host insect, which has evolved to synthesize proteins at high levels to allow the insect larvae to undergo growth and development in a very compact time frame.

Whereas equally high levels of gene expression are present in other eukaryotic systems, e.g., the production of ovalbumin in the avian oviduct and the production of various milk proteins in mammals, the expression of these proteins is limited to specific tissues and does not occur systemically. In addition, whereas transgenic animals have been engineered to exploit these systems, they can in no way compare with the

ease of manipulating baculoviruses and their cell culture systems for protein production.

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Chapter 11
Baculoviruses, retroviruses, and insect cells

There are a variety of classes of transposable elements (TEs) integrated into the genomes of all cells and they are often major components of cellular genomes. For example, from 0.3% of bacterial genomes up to almost 80% of some vertebrate genomes are composed of these elements (reviewed in [1]). In addition to DNA transposons, many TEs are retroelements, which have an RNA intermediate. They often encode a reverse transcriptase, which can convert the RNA of the retroelement into cDNA that is then integrated into the host cell genome. A major category of retroelements includes the retroviruses that are infectious and can spread between organisms. The genome of the retrovirus becomes integrated into a host genome and is called a provirus and contains long terminal repeats (LTR) at either end that encode regulatory elements, a gag gene that encodes a structural (capsid) protein, the polymerase gene that encodes several enzymatic functions, and the envelope gene, env, that provides the virus with the ability to infect other cells (Fig. 11.1A). There are several different categories of retroelements; some lack LTRs, while others lack an env gene, and are normally confined to a cell and are not infectious. In addition, some do not encode a reverse transcriptase, but rely on other retroelements to supply this enzyme. The focus of this chapter is on a group of retroelements found in insects that resemble retroviruses.

A baculovirus-associated errantivirus (retrovirus)

Few polyhedra (FP) mutants are a readily observable baculovirus phenotype that result in reduced numbers of polyhedra and an elevated titer of budded virus. Such mutants often contain an insert in the *fp* gene (ac61), although the phenotype can result from mutations elsewhere in the genome. In the process of characterizing AcMNPV FP mutants produced after 25 passages in *Trichoplusia ni* cells, an isolate, (FP-D), was found to contain an integrated retrotransposon that originated from the host genome. It was called transposable element D or TED [2]. This element had features of a retrovirus including long terminal repeats and was demonstrated to express gag, pol, and env-like genes that are capable of being incorporated into virus-like particles [3-5]. Normally retroviruses that integrate into a genome remain integrated and are spread and amplified by the RNA intermediate that is transcribed from the integrated provirus by the host cell RNA polymerase II. In contrast, the TED provirus was found to be unstable and upon excision, a copy of one LTR of about 270 bp remained in the baculovirus genome [6]. This instability was probably due to the large size of the TED genome relative to the baculovirus genome. Viruses related to TED have been found in other insects with the retrotransposon called *gypsy* from *Drosophila* being the most well studied example.

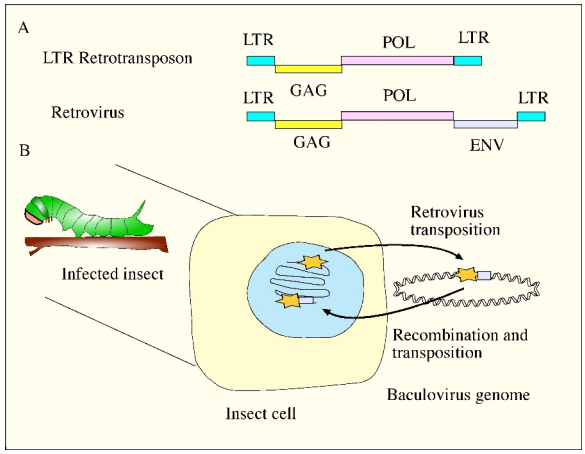

Fig. 11.1. The conversion of a LTR retrotransposon to a retrovirus by obtaining the env gene. A. The difference between LTR retrotransposons and retroviruses is the env gene. B. A theoretical diagram of the incorporation of a LTR retrotransposon into a baculovirus genome adjacent to the envelope fusion protein. By DNA recombination, the DNA would have incorporated the envelope fusion protein and then transposed into the genome of an insect cell.

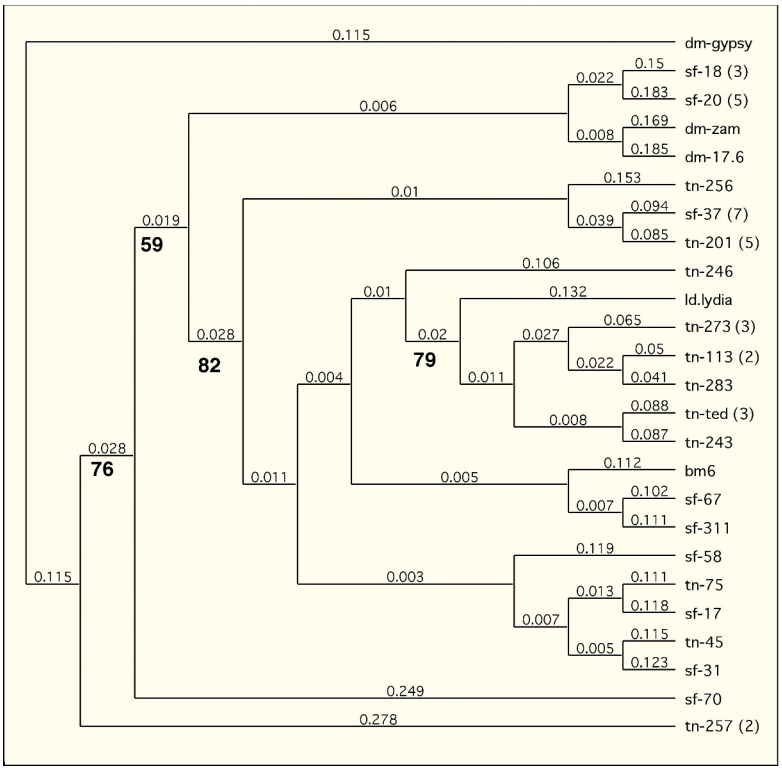

Fig.11.2. Phylogenetic tree of *S. frugiperda* and *T. ni* errantivirus sequences. The sequences were PCR amplified from the reverse transcriptase region. Nodes with bootstrap values greater than 50% are indicated as bold numbers. The numbers in brackets indicate the number of times the sequence was found in the data set. Bm6 is from *B. mori*, Ld-lydia is from *L. dispar*, and dm-gypsy, dm-17.6, and dm-zam are from *Drosophila*. For details see [11].

The insect retroelements that encode an env gene are called the errantiviruses (from Latin *errans*, to wander). Although similar to retroviruses, they have not been included within the Retroviridae because they are a distinct lineage, and evidence that they are infectious is indirect (see below). Kanga and roo-like retroviruses also encode a related env gene [7]. These retrovirus-like elements are often found in multiple copies and are present as apparent complete and truncated or defective genomes. For example in the *Drosophila melanogaster* genome there are five different categories of errantiviruses encompassing 78 complete or partial sequences that range from a single full length copy of *gypsy* to 18 full length/ 39 partial copies of the element *297*. Other categories include *17.6* (7 full length/5 partial) and *idefix* (2 full length/5 partial). A fifth category, *zam*, was not found in this sequence indicating the variability of errantivirus distribution between *D. melanogaster* strains [8].

Errantiviruses in Lepidoptera

Two of the main cell lines used for baculovirus research were derived from primary explants of pupal ovarian tissue from moths of the family Noctuidae. One is from the fall army worm (*Spodoptera frugiperda* (Sf)) [9] while the other is derived from the cabbage looper, *Trichoplusia ni* (Tn), [10]. In a survey of the genomes of these cell lines, using degenerate oligomers targeted to a conserved region of the errantivirus reverse transcriptase gene, over 20 different PCR products from each cell line were amplified, cloned, and sequenced. Analysis of these sequences resulted in the identification of over 20 lineages that could be grouped into several major clades (Fig. 11.2). Three of the sequences were identical to the TED errantivirus described above [11]. Phylogenetic analyses indicated that most of the Sf and Tn sequences were closely related to each other and to sequences from other Lepidoptera. The next most closely related sequences were from the *Drosophila* (Diptera). However, there are several sequences from both Sf and Tn that form lineages distinct from the majority of the lepidopteran or dipteran sequences.

Relationships between insect retroviruses and baculoviruses: the env gene

Phylogenetic analyses of the errantivirus env gene and baculovirus genome sequence data

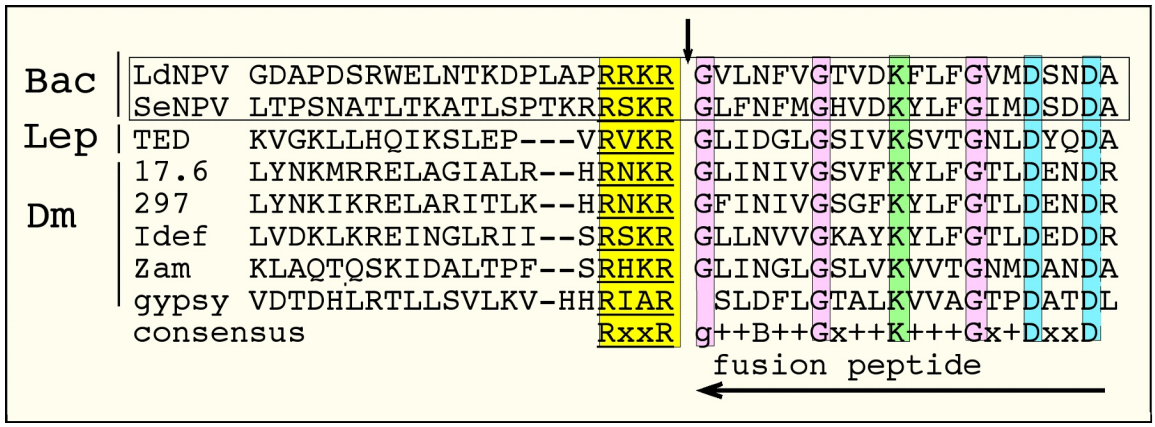

Fig. 11.3. Homology at the cleavage site of selected baculovirus F proteins, a lepidopteran (Lep) errantivirus env [61], and 5 env sequences from *Drosophila melanogaster* (Dm). The two sequences at the top (in box) are from baculoviruses (Bac). The furin cleavage signal (RxxR) is underlined, and colored yellow. The vertical arrow shows the furin cleavage site. Other conserved amino acids are also colored. The predicted fusion peptide is indicated by the horizontal arrow. In the consensus sequence hydrophobic amino acids are indicated by (+), N or D by (B), and variable amino acids by (x). For more information see [13].

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resulted in the unexpected observation that the errantivirus env gene is related to the baculovirus envelope fusion protein, or F protein lineage [12, 13] [14]. The sequence similarity was most striking in the region that includes the furin cleavage signal (RxxR) and a predicted fusion peptide immediately downstream.

This fusion peptide of about 20 amino acids is highly hydrophobic except for 2 D and 1 K residue (Fig. 11.3). The errantiviruses most likely obtained the F protein from a recombination event that occurred when a retrotransposon integrated into a baculovirus genome (Fig. 11.1). Evidence for such an event is compelling because the errantivirus TED was found integrated into a baculovirus genome as described above [2]. Baculoviruses have two different envelope fusion proteins; gp64 and F. GP64 appears to have been recently incorporated into one baculovirus lineage called Group I. Whereas the Group I baculoviruses retain a copy of the F gene, it no longer functions independently as a fusion protein. In contrast, most baculoviruses (Group II) lack gp64 and contain only a copy of the F gene suggesting that it is the fusion protein in these viruses (reviewed in [15]) (see Chapter 2).

In addition to the strong evidence for the capture of a baculovirus envelope fusion protein leading to the evolution of errantiviruses, this phenomenon appears to be a relatively common event in virus evolution and may have occurred a number of times [12, 16]. It has not only occurred with elements such as retrotransposons which commonly integrate into DNA, but also has been observed for a variety of other categories of viruses including members of the Orthomyxoviridae and with the baculovirus gp64 gene described above (reviewed in [15]).

Cellular homologs of baculovirus F/errantivirus env proteins

In addition to the relatedness of the baculovirus F and errantivirus env genes, a cellular homolog in the *Drosophila* genome sequence was also identified [13]. However, this protein is not cleaved (see below), does not have membrane fusion activity, and appears to localize to intracellular organelles rather than cell membranes [17]. This gene was determined to have entered the Drosophila lineage once and another time into a mosquito lineage. In *Drosophila* it is expressed in most tissues analyzed in both adult males and females. It was suggested that it was incorporated into and retained by the insect genome because its expression could protect the host cell from infection by retroviruses or baculoviruses that shared a related env protein. This could be accomplished if the cellular homolog binds to and interferes with the viral receptors on the cell

surface or if they act as dominant negative inhibitors in which the endogenous env would complex with and inactivate the viral env protein [7].

Features of baculovirus F and insect retrovirus env proteins.

The baculovirus F and errantivirus env proteins appear to be members of a group of envelope fusion proteins common to many vertebrate viruses. Although, in general, they lack convincing sequence relatedness, it has long been suggested that a number of envelope fusion proteins from a variety of disparate viruses are related. This is based on their requirement for cleavage to be activated and the fact that one of the resulting peptides is membrane associated via a transmembrane domain. In addition, the membrane-associated peptide contains a hydophobic fusion peptide domain downstream of a cleavage site followed by predicted coiled-coil domains that are involved in forming hairpin-like structures that are important in virus-cell fusion [18, 19]. Such structures have been characterized in fusion proteins from viruses as diverse as filoviruses, retroviruses, orthomyxoviruses, and paramyxoviruses. Evidence suggests that baculovirus F proteins are members of this group (Fig. 11.3), and it has been demonstrated that they require cleavage, most likely by the host cell furin protease, for activation [20, 21]. Errantivirus env proteins also have similar features [13] consistent with their being members of this group. Furthermore, *gypsy* env accumulates at the cell membrane as would be expected for a viral envelope protein and contains a predicted furin cleavage site and it is cleaved when expressed in both *Drosophila* S2 and Sf9 cells [22].

Additional relationships of insect retroviruses and baculoviruses.

The relatedness of the errantivirus *env* protein and the baculovirus *F* homologs may reflect more than a fortuitous recombination event between these two viruses. The errantivirus TED is a mid-repetitive element (about 50 copies/genome) in *Trichoplusia ni* [6] and is capable of transposition from the insect into the baculovirus genome. A key feature of the relationship that may have led to the capture of a baculovirus F gene by a pre-errantivirus retrotransposon involves the ability of baculoviruses to express genes at very high levels. This feature appears to be due at least in part to the fact that they encode an RNA polymerase [23] capable of high levels of transcription in the context of the virus replication program. This polymerase recognizes a unique promoter sequence (A/G/TTAAG) [24] that is found in the TED LTR as a palindrome. Late in the baculovirus infection, mRNA is expressed from these LTRs at high levels [6]. Therefore, integration into a baculovirus genome may reflect a strategy to exploit baculovirus late gene expression to express the integrated retrotransposon/retrovirus genome at high levels. This could result in the production of a mixture of retrovirus particles and occluded baculoviruses containing integrated retroviruses and would provide two methods of escape from an insect with a fatal baculovirus infection: they could either survive by integration into baculovirus genomes, or possibly as infectious virus particles (Fig. 11.4). The evolution of this relationship between a baculovirus and a primordial LTR-type retrotransposon provides a clear pathway, via DNA recombination, for the transposable element to incorporate the baculovirus F homolog into its genome, thereby converting it into a potentially infectious retrovirus (Fig. 11.1B).

Are errantiviruses infectious?

Early on it was noted that retrovirus like particles and reverse transcriptase activity were present in *Drosophila* cells [25]. Subsequently, *gypsy* became the most intensively studied retrovirus-like element in in *D. melanogaster*. Indirect evidence suggests that *gypsy* is infectious for *Drosophila* [26, 27]. These data were obtained by feeding a strain of *Drosophila* that lacks active *gypsy* transposition with either purified vlps from insects with transpositionally active *gypsy*, or extracts derived from such insects, and then documenting increased levels of transposition in the recipient insects. Similarly, it was observed that the *gypsy* could be transmitted between cells in culture [28].

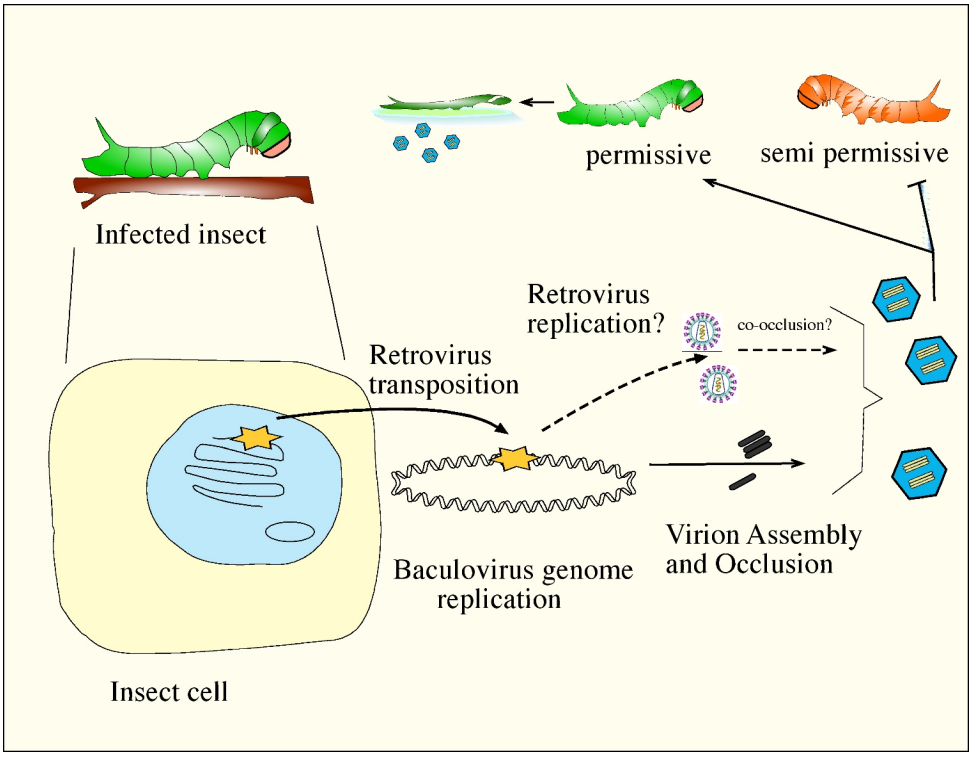

Fig. 11.4. Possible pathways of retrovirus survival and transmission in a baculovirus infected host. A retrovirus integrated into an insect genome might have two mechanisms for surviving a fatal infection. It could transpose into a baculovirus genome and as a provirus become incorporated into a baculovirus virion and subsequently occluded. As a provirus it could likely remain stable in the occlusion body indefinitely. The other mechanism is more theoretical and is shown by dashed arrows. Retrovirus could be produced from the provirus in the baculovirus genome. Retrovirus genomic RNA would be produced from the late promoter elements in the LTR, these could be translated into proteins and mature virions could be produced. These could also be occluded in occlusion bodies and would likely be stable similar to the baculovirus virion. Upon encountering an insect semi permissive for virus infection, the retrovirus could invade the new host during abortive replication.

Does env play a role in errantivirus infectivity?

The evidence implicating env in errantivirus infectivity is varied. In one study, it was found that a preparation of two monoclonal antibodies against *gypsy* env mixed with the vlp fraction, reduced the number of insertion events in insect feeding experiments [29]. In addition, evidence suggests that an integrated Moloney leukemia virus-luciferase construct pseudotyped with a *gypsy* envelope is infectious for *Drosophila* cells [30]. This suggested that *gypsy* env is capable of mediating infection of *Drosophila* cells. Although *gypsy* may be infectious, its infectivity appears to be very limited. It has been suggested that since they are adapted for integration into the cell genome, they no longer require propagation via infection [31]. However, this does not explain why they have retained, conserved, and continue to express an *env* gene. Since envelope proteins often play major roles in the virulence of other viruses [32], the errantivirus *env* proteins may allow the viruses to be infectious and spread between different organisms, but this infectivity may be restricted by features of their env proteins. It has also been suggested that *gypsy* env has fusogenic properties [33].

The invasion and amplification of retroelements

The baculovirus associated errantivirus, TED, described above that was originally found

integrated into the AcMNPV genome is a particularly useful example of how a virus could spread a transposable element to other species. One can imagine a situation in insects where a baculovirus abortively infects a species that might be semi permissive for virus replication (Fig. 11.4). The infection could result in low levels of baculovirus replication that would allow mRNA expression, cDNA production and transposition of a TE from the invading baculovirus into host cell DNA, but the host would eventually overcome and survive the baculovirus infection. An example of such virus/insect combinations could be AcMNPV pathogenesis in *Helicoverpa zea* which is over 1000 fold more resistant to fatal infection than *Heliothis virescens* [34]. The primary midgut infection and secondary infection of the tracheal epidermis are similar in the two insects, but *H. zea* larvae are able to encapsidate infected cells thereby limiting the infection. Under these conditions, the surviving insects could have been exposed to and possibly parasitized by a TE carried by the invading baculovirus, whereas in contrast, the carrier baculovirus would have been eliminated.

Once a retroelement integrates into a genome, they are transcribed into RNA, reverse transcribed into cDNA that can integrate elsewhere in the genome. Via this process they can greatly amplify their copy number and the size of the genome of their host organism. Depending on the element, some TEs can transpose at a rate of from 10^{-3} to 10^{-5} per element per generation. Consequently they can be more significant in the production of genetic change that normal nucleotide base changes which are altered at about 10^{-8}–10^{-9} per nucleotide per generation. Bursts of transpositional activity are thought to have been a major force in the evolution of new species (reviewed in [1]). For example, the activation of a gypsy-like retrotransposon in plants of the genus Gossypium, which includes cotton, is responsible for a three fold difference in the genome size between some species. Similarly, the amplification of several TE families has led to the doubling of the size of the rice genome. These elements often accumulate in heterochromatic regions of the genome. Such regions normally contain repetitive DNA and are transcriptionally inactive and include centromeres and telomeres (reviewed in [35]).

What prevents retroelements from amplifying continuously?

The insertion of transposable elements can interrupt genes, alter regulatory regions such as promoters and enhancers, and disrupt patterns of splicing and 3' transcriptional processing and therefore can be highly mutagenic. Mutations in germ line cells could affect the success of progeny. In somatic cells, mutations could cause localized disruption of cell function, or they could cause more generalized effects if they alter the regulation of functions such as cell division that could lead to the production of oncogenic cells. Consequently, molecular systems have evolved to defend cells against TEs. Therefore, although molecular evidence suggest that amplification of TEs is a major feature of eukaryotic genome evolution, most are eventually silenced by the host. If a lineage survives the invasion and amplification of a TE, at some point its further proliferation is quelled. This is accomplished by several epigenetic mechanisms that involve inherited processes that do not affect the DNA sequence. These include post-transcriptional silencing by RNAi, chromatin modifications by changes in DNA methylation patterns, histone modifications including methylation, and changes in chromatin condensation and packing. It is likely that these processes work cooperatively to both suppress transcription and to eliminate RNA that is expressed.

Suppression of transposable elements by DNA and histone methylation

Although endogenous retroviruses are normally silenced, their active transcription can be detected at different stages during development. For example, a number of retroelements in Drosophila show patterns of spatially regulated expression during embryogenesis [36]. DNA methylation involves the addition of a methyl group to the cytocine in CpG sequences. CpG sequences are often found concentrated in 'islands' present in promoter regions. Such regions are often characterized by increased histone occupancy with a corresponding reduction in the binding of transcription factors. Methylated DNA can also attract methyl-binding proteins that also can inhibit transcription. It has been suggested that one of the primary roles for this phenomenon is to prevent transcription of TEs thereby protecting the host from

endogenous retroviruses (reviewed in [37, 38]). Although some insect genomes are highly methylated, the patterns of these modifications may differ from other organisms. It may be associated with non-CpG dinucleotides and may not be focused on mobile sequences that are often heavily methylated in the genomes of vertebrates and plants [39, 40]. Transcription can also regulated both positively and negatively by the pattern of histone methylation [41].

Suppression of transposable elements by RNA interference

Three pathways involved in RNA silencing have been identified in both mammals and insects and a major focus of these pathways is the suppression of endogenous transposable elements. These pathways include: i) RNA interference (RNAi) that employs small interfering RNAs (siRNAs) that are derived from exogenous double stranded RNA (dsRNA) and can act as a defense against viral infection by targeting TE RNA for degradation. In addition, siRNAs can also be derived from endogenous sequences and are involved in suppressing the expression of endogenous transposable elements in somatic cells; ii) microRNAs (miRNAs) involve endogenous small RNAs that repress partially complementary mRNAs[42]; and iii) Piwi-interacting RNAs (piRNA) that repress transposons in germ line cells and can also activate transcription in heterochromatin which is a gene-poor, highly condensed, DNA-protein complex (reviewed in [43]). Therefore both exogenous and endogenous RNAs can be inactivated and different mechanisms can be involved in germ line and somatic cells.

The Argonautes: proteins with RNAse activity that are critical in RNA interference

Key to the function of interfering RNAs is their interaction with Argonaute proteins [44]. Although in Greek mythology the Argonautes were sailors on the ship Argo who accompanied Jason in his search for the Golden Fleece, in molecular biology the term was originally used to describe the shape of the leaves of a mutant of *Arabidopsis thaliana*, *AGO1*, because they resembled the squid *Argonauta argo* [45]. The Argonaute proteins facilitate both the processing of some micro RNAs by eliminating the non-active siRNA strand and also use small RNAs as guides to identify and repress complementary transcripts by degradation (via endonuclease activity) or by inhibition of translation. They appear to have evolved from the RNAse H family of endonucleases but have substituted ssRNA for ssDNA as the template to target RNA (reviewed in [44]*).*

Suppression of transposable elements in gonadal cells

A major category of piRNAs include rasiRNAs (repeat associated siRNAs) that are involved in the silencing of transposable elements. PiRNAs map to repetitive elements throughout the *Drosophila* genome, however a limited number of loci called piRNA clusters appear to match most piRNAs. The transposons in the clusters involved in piRNA production appear to be truncated or defective relicts and are probably not capable of autonomous expression or transposition. It has been suggested that piRNAs are derived from long single stranded precursor RNAs in which a 5' cleavage occurs at a uridine residue. The sequence then becomes incorporated into a Piwi protein complex where a second cleavage occurs generating the specific size. The piRNA then targets the piwi complex to RNA expressed from transposable elements. Further evidence for the role of Piwi type proteins in the suppression of transposable elements in gonadal tissue was the observation that a mutation in piwi reduced the repression of gypsy in restricted tissues leading to up to a 150 fold increase in gypsy RNA levels [46, 47]. PiRNAs have also been described from Bombyx mori and they appear to include a major subclass of rasiRNAs and are thought to be involved in transposon silencing and development of germ line cells [48, 49] [50].

In *Drosophila*; *flamenco*, a source for piRNAs

A locus called *flamenco* controls the activity of retroviral elements *gypsy* [51], *idefix*, and *zam* [52]. The *flamenco* locus was mapped to a region that corresponds to a piRNA cluster spanning a region of 179 kb and is comprised of nested transposable elements and fragments including those specific to *gypsy*, *idefix* and *zam*, in addition to other transposable element-specific sequences. Such regions are called piRNA clusters and essentially lack protein

coding sequences and are comprised of truncated or damaged copies of TEs that appear to lack the capacity to be mobilized and are concentrated in the pericentromeric or telomeric heterochromatin [47, 53]. Other loci that are involved piRNA production have also been described [54, 55].

Summary: unanswered questions

Retroelements have had a major influence on genome expansion and evolution in eukaryotes. Complex systems including siRNA described above have evolved to control their expression. The source of this siRNA has been mapped to regions containing extensive sets of repeated fragments of TEs that can be used for the generation of siRNA specific to major categories of TEs. Therefore, the apparent extensive past amplification and integration of multiple copies of TEs has in itself contributed to the eventual suppression of their further proliferation. In addition, the patterns of methylation of both DNA and histones can also suppress the expression of TEs.

Since it is likely that the TEs of Lepidoptera are regulated in a similar manner to those described in other eukaryotes, a major question that arises with regards to baculoviruses is the extent to which infection by these viruses might relax or inhibit the silencing systems present in their host cells thereby facilitating the amplification of endogenous TEs. Viruses have been demonstrated to interfere with RNAi systems in a variety of organisms [56]. Although it is likely that baculoviruses also have evolved mechanisms to resist host cell programs of RNA based interference, the presence of secondary effects that might facilitate transposition of endogenous retroviruses such as TED might not have had a compelling selective advantage for the baculovirus. However, there are a number of non specific effects of baculovirus infections that might favor endogenous retrovirus replication and transposition. For instance it is well documented that transcription of many RNA polymerase II transcribed genes is turned off as the baculovirus infection progresses (see Chapter 4 and 10) [57-59]. It has also been shown that RNA polymerase II transcripts of viral mRNA are degraded late in infection, e.g. see [60]. This could interfere with RNAi production if it was carried out by this polymerase. How these characteristics of baculovirus infection might affect the RNA pol II transcripts derived from the integrated provirus such as TED [61] remains to be examined.

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Chapter 12

The AcMNPV genome: Gene content, conservation, and function

The *Autographa californica* nucleopolyhedrovirus (AcMNPV) was originally described in the early 1970s (1) and research on its genetics began later in that decade. This was stimulated by the facility with which the virus replicated in cells from *Spodoptera frugiperda* (2) and *Trichoplusia ni* (3). Subsequently, this led to the development of technology for the deletion of genes (4, 5), and allowed for targeted studies on the function of specific genes, particularly if the deletion or mutation of the target gene was not lethal to the virus. The publication of the genome sequence of AcMNPV (6) was a landmark in the investigation of this virus because it put all previous and future investigations into context. It also revealed genes that are shared with other organisms, and provided the basis for understanding baculovirus diversity. Subsequently, the development of the bacmid system, which allowed for the production of recombinants via transposition of recombinant plasmids into the AcMNPV genome incorporated into a bacterial artificial chromosome, allowed for manipulation of the whole AcMNPV genome in bacteria (7). The adaptation of this technology to making targeted deletions provided a method for constructing baculovirus gene knockouts in bacteria that could then be investigated via transfection into insect cell lines (8). The modification of this technology using a lambda red recombinant system (9) allowed for the more efficient production of knockouts (10). This has resulted in a proliferation of studies on AcMNPV essential genes that were previously difficult to investigate because knockouts were lethal and they could not be readily generated without the production of complementary cell lines. With bacmid knockouts the function of the target gene can be inferred from the examination of cells transfected with DNA of the mutant. The adaptation of similar bacmid systems for other baculovirus genomes has allowed for parallel studies on these viruses. Concomitant with the development of techniques for investigating gene function has been a proliferation of complete genome sequence data for many baculoviruses. Because of the widespread use of AcMNPV, not only for fundamental investigations on gene function, but also because of its use as an expression vector, I have attempted to annotate the AcMNPV genome in terms of understanding the function of each of the genes.

Adjustments to the AcMNPV genome sequence: There are approximately 150 orfs

Several regions of the genome of the AcMNPV C-6 strain have been resequenced by one or more laboratories. This, in combination with genome sequences of variants of AcMNPV that have become available, has revealed revisions that should be incorporated into the sequence. The following summarizes the physical corrections to the AcMNPV genome that have been described. The original orfs Ac20/21, 58/59, 106/107 and 112/113 (6) should be combined into single orfs. Ac20/21 was resequenced when it was implicated in actin rearrangement (11), and all four of these regions were resequenced as a comparison of the AcMNPV and RoMNPV genomes (12). In addition, resequencing also indicted that Ac17, 52, 131, and 143 are longer than originally reported (13). The total number of orfs reported for AcMNPV was originally calculated to be 154; this adjustment reduces it by 4. However, the discovery of lef-10 (Ac53a), which was missed in the original annotation because of its significant overlap with the 5' region of an adjacent gene (Ac54) (14), adds an additional orf. Therefore, there appear to be 151 orfs present in the genome based on the original criteria of an orf comprising 50 amino acids. However, Ac85 (53 aa) is only found in two other NPVs that are variants of AcMNPV (PlxyNPV) (13) and RoMNPV (12). Two orfs, Ac97 (56 aa) and Ac140 (60 aa), are only present in AcMNPV. Of these three orfs, only Ac97 was predicted to be preceded by transcriptional regulatory sequences (6). Although these three orfs may

not be valid, others may be present that have not yet been detected. Therefore, it would appear that there are 'about' 150 orfs in the AcMNPV genome.

In this review, I have attempted to include pertinent information on the function of all the orfs present in the AcMNPV genome. This is not an exhaustive review, but an attempt to infer function from either actual experimentation on the AcMNPV gene or to homology from related viruses. A significant proportion of the data included comes from the *Bombyx mori* NPV (BmNPV). It is closely related to AcMNPV and is the second most studied NPV. In addition, an extensive program of deletion mutagenesis was undertaken on BmNPV (15). From my own experience, I have found this data to be highly accurate. However, because the bacmid protocol was not available, paired repair viruses were not produced for most of these constructs. In addition, when deletions were lethal the result was negative and no further experimentation could be conducted. In this survey, I have found that there is deletion, or in a few cases ts mutation, data for about 40% (~65) of the genes in the AcMNPV genome. There is further data for a similar number that have been deleted only in BmNPV. The remaining BmNPV or AcMNPV genes for which there is no deletion data are either not present in BmNPV, or not identified in BmNPV because the cut-off for analysis was 60 aa in BmNPV (16), vs. 50 aa in AcMNPV (6). In addition, for three genes, deletion data is unavailable for BmNPV.

Table 1 lists all the genes that have been named in the AcMNPV genome followed by the orf number. It cannot be emphasized enough how important it is to incorporate both orf numbers and the AcMNPV homolog (if present) into any description of a baculovirus gene no matter which baculovirus it is from. Without this reference, it is difficult, if not impossible, to put reports into any sort of perspective. Following the index is a review of what I could find regarding all the genes in the AcMNPV genome. This is not a complete review of these genes but focuses particularly on AcMNPV. When a report of the homolog from another virus is relevant, that material is also included. The data in this summary was greatly facilitated by the Viral Bioinformatics Resource Center web site that allowed for the convenient comparison of baculovirus genes and genomes (17).

Table 1. Index to named AcMNPV genes/gene products

ARIF1 (Actin rearranging factor1)	**Ac20/21**
Alkaline nuclease	**Ac133**
BRO (Baculovirus repeated orf)	**Ac2**
BV/ODV-E26	**Ac16**
BV/ODV-C42	**Ac101**
Calyx, polyhedron envelope	**Ac131**
Cathepsin	**Ac127**
ChaB homolog	**Ac59**
ChaB homolog	**Ac60**
Cg30	**Ac88**
Chitinase	**Ac126**
Conotoxin like (Ctl)	**Ac3**
Desmoplakin-like	**Ac66**
DBP (DNA binding protein)	**Ac25**
DnaJ domain protein	**Ac51**
DNA polymerase	**Ac65**
EGT	**Ac15**
ETL (PCNA)	**Ac49**
ETM	**Ac48**
ETS	**Ac47**
Exon-0	**Ac141**
F (fusion protein homolog)	**Ac23**
FGF (fibroblast growth factor)	**Ac32**
FP (few polyhedra)	**Ac61**
Fusolin (gp37)	**Ac64**
GP16	**Ac130**
GP37	**Ac64**
GP41	**Ac80**
GP64	**Ac128**
Gta (global transactivator)	**Ac42**
Hcf-1 (host cell factor 1)	**Ac70**
Helicase, p143	**Ac95**
He65	**Ac105**
Homologous regions	**Hrs**
Iap-1	**Ac27**
Iap-2	**Ac71**
Ie1	**Ac147**
Ie0	**Ac147-0**
Ie2	**Ac151**
Lef1	**Ac14**
Lef2	**Ac6**
Lef3	**Ac67**
Lef4	**Ac90**
Lef5	**Ac99**
Lef6	**Ac28**
Lef7	**Ac125**
Lef8	**Ac50**
Lef9	**Ac62**
Lef10	**Ac53a**
Lef11	**Ac37**
Lef12	**Ac41**
Me53	**Ac139**
MTase (methyl transferase)	**Ac69**
Nudix	**Ac38**
ODV-E18	**Ac143**
ODV-E25, p25, 25k	**Ac94**
ODV-EC27	**Ac144**
ODV-E56	**Ac148**
ODV-E66	**Ac46**
P6.9	**Ac100**
P10	**Ac137**
P12	**Ac102**
P24	**Ac129**
P26	**Ac136**
P33	**Ac92**

P35	**Ac135**
P43	**Ac39**
P45, p48	**Ac103**
P47	**Ac40**
P74, pif	**Ac138**
P94	**Ac134**
PCNA	**Ac49**
Pe38	**Ac153**
Pif-1	**Ac119**
Pif-2	**Ac22**
Pif-3	**Ac115**
Pif-4	**Ac96**
Pif-5	**Ac148**
PK1 (Protein kinase 1)	**Ac10**
PK2 (Protein kinase 2)	**Ac123**
PKIP (Protein kinase interacting factor)	**Ac24**
PNK polynucleotide kinase	**Ac33**
PNK/PNL polynucleotide kinase/ligase	**Ac86**

Polyhedrin	**Ac8**
Pp31;39K	**Ac36**
Pp34, polyhedron envelope	**Ac131**
Pp78/83;orf1629	**Ac9**
Protein tyrosine phosphatase (ptp)	**Ac1**
SOD superoxide dismutase	**Ac31**
Sulfhydryl oxidiase, sox	**Ac92**
TLP telokin-like	**Ac82**
TRAX-like	**Ac47**
Ubiquitin	**Ac35**
VLF-1 very late factor 1	**Ac77**
Vp39, capsid	**Ac89**
Vp80, vp87	**Ac104**
Vp91	**Ac83**
Vp1054	**Ac54**
19K	**Ac96**
38K	**Ac98**
49K	**Ac142**

Annotation of the AcMNPV genome

Below is an annotation of the orfs in the AcMNPV genome based on their orf number from (6). An asterisk (*) indicates a core gene with homologs found in all sequenced baculovirus genomes. The orf size in amino acids, followed by the molecular mass in kDa is indicated after each orf as reported by (6, 12). The same information is also included for the BmNPV orthologs of these genes. The distribution of the genes is from analyses completed in early 2008; therefore, the viral distribution reflects the genomes available at that time. The survey of information regarding each gene was updated late 2010. Another similar source for this information is (18).

Ac1 (168aa:19.3kDa), (Bm130:168aa:19.3kDa), (protein tyrosine phosphatase (ptp); baculovirus phophatase (bvp)). Ac1 homologs are present in the genomes of all lepidopteran Group I NPVs, but not those of other baculoviruses. Closely related orfs are found in a variety of invertebrates, e.g., *Drosophila* (E = 7e-30) and vertebrates, e.g., human (E = 2e-27). It is expressed from a late promoter (19). It was originally identified because of its relatedness to protein tyrosine phosphatases and its ability to dephosphorylate proteins at ser, thr and tyr residues (20). However, it was later found that Ac1 is an RNA 5'-triphosphatase and hydrolyzes the gamma phosphate of triphosphate-terminated poly(A) and also hydrolyzes ATP to ADP and GTP to GDP (21, 22). The crystal structure has been determined (23). Ac1 is predicted to have one of the same enzymatic activities of LEF-4 (Ac90) that is involved in preparing RNA for cap formation. Although deletion is not lethal, mutants are partially defective in occluded virus production in Sf-21, but not Tn-368 cells (24). During the final larval stages many Lepidoptera disperse (wander) probably as an evolutionary mechanism to spread the population, reduce predation, and find an optimal location for pupation. Baculovirus infection appears to be capable of enhancing this behavior possibly as a mechanism of spreading the virus. It was found that BmNPV deleted for the tyrosine phosphatase gene failed to undergo this enhanced wandering behavior (25). It is not clear whether the enzymatic activity of this protein is related to this observation. Although not reported to be associated with ODV by proteomic analysis, it was found in BV preparations (26).

Ac2 (328aa:38.8kDa),(Bm131:349aa:40.1kDa), (Baculovirus repeated orf—BRO). *Derivation: In American inner-city dialect 'bro' means 'brother', but not necessarily a close relationship*.

Homologs of Ac2 have a widespread distribution in lepidopteran NPVs and GVs and are also found in the dipteran, but not hymenopteran NPV genomes. Related orfs are also found in double-stranded DNA phage, prokaryotic class II transposons, and a variety of DNA viruses pathogenic for insects, including entomopox viruses, iridoviruses and ascoviruses (27). Twenty-three copies of *bro* genes have been reported in a *Heliothis virescens* ascovirus genome sequence (28). Although there is only one copy of the *bro* gene in AcMNPV, the number can vary in different baculoviruses from none in the closely related *Rachiplusia ou* MNPV — its orfs are 96% identical in sequence to AcMNPV (12), two in the more closely related PlxyNPV (13) (orfs are 98.5% identical to AcMNPV), to up to 16 copies in LdMNPV (29). AcMNPV Ac 2 deletion mutants are viable, but some differences in polyhedron production in infected cells were noted (27). One of the major differences in gene content between BmNPV and AcMNPV is the presence of five copies of *bro* genes in BmNPV vs. a single copy in AcMNPV (16). The BmNPV *bro* genes are present in three locations with duplicate genes in two of the locations. In contrast, another BmNPV strain only shows 3 copies of the gene, one at each of the three locations (30). This suggests that duplication/loss of these genes might be common. In the strain with five genes, individual mutants of four of the genes were isolated in BmNPV, but a mutant of one gene (*bro-d*) could not be isolated. Also, a double mutant of *bro-a* and *bro-c* could not be produced, suggesting that they complement an essential function and that *bro-d* has a unique essential function (31). BmNPV bro proteins have DNA binding activity (32), and all the BmNPV *bro* genes appeared to be expressed as early genes and are distributed in both the nucleus and cytoplasm (31). One of the *bro* gene products was found to interact with laminin, a

glycoprotein that is a major constituent of the basal lamina and is involved in cell attachment (33).

Ac3 (53aa:5.6kDa) (Conotoxin-like (*ctl*) genes). Conotoxins are small disulfide-rich ion channel antagonists isolated from snails (genus *Conus)* (34). Homologs of *ctl* are found in about half of the sequenced Group I and Group II lepidopteran NPV genomes and two GVs (Xcni- and HaGV). Although in AcMNPV a single *ctl* gene is present, several other viruses, e.g., OpMNPV and LdMNPV, encode two *ctl* genes of different lineages (*ctl-1* and *ctl-2*). Homologs are found in the *Amsacta moori* entomopoxvirus (E = 0.006), a few mosquito species, a funnel web spider, a wasp, and a bacterium. The EPV (*A. moori* gene falls within the baculovirus *ctl-2* lineage. In a study examining the AcMNPV *ctl* (*ctl*-1) gene, no differences in mortality, motility, or weight gain were observed when either neonate or late instar *Spodoptera frugiperda* larvae were infected with an AcMNPV mutant deleted for *ctl-1*, compared with infection with wt virus (35).

Ac4 (151aa:17.6kDa), (Bm133:151aa:17.7kDa). Homologs of Ac4 are found in all Group I, three group II, and two GV genomes, but not in other lineages. Ac4 is likely to be nonessential because when it was interrupted by insertional mutagenesis in BmNPV (Bm133), the virus appeared to be viable (15).

Ac 5 (109aa:12.4kDa), (Bm134:109aa:12.4kDa). Homologs of Ac5 are found in the genomes all Group I (except AnpeNPV) NPV genomes. It may be ODV associated (36). Ac5 is likely to be nonessential because when it was interrupted by insertional/deletion mutagenesis in BmNPV (Bm134), the virus appeared to be viable (15).

***Ac6 (210aa:23.9kDa), (Bm135:210aa:23.8kDa) (Lef-2).** LEF-2 is a DNA primase accessory factor and is encoded by all baculovirus genomes. It interacts with LEF-1 (37), the baculovirus DNA primase. It has homology to the large subunit of DNA primase in several archaea. It is required for transient DNA replication (38, 39). Based on limited amounts of DNA synthesis by a *lef-2* bacmid knockout in transfected cells, it was suggested that Lef-2 is not required for the initiation of DNA replication. This was in contrast to a bacmid with the helicase gene deleted that showed no synthesis (40). However, the transfected DNA is likely nicked and therefore the ends of the nicks could act as primers, resulting in limited amounts of leading strand DNA synthesis in the absence of an active primase complex. Elution profiles of LEF-1 and LEF-2 from ssDNA cellulose and DEAE resin, suggested that LEF-2 may bind to both DNA and LEF-1 (41). LEF-2 mutants have been characterized that appear to affect very late transcription, indicating that it may have roles in both replication and transcription (42). In addition, BmNPV LEF-2 was shown to activate late transcription (43). It is an essential gene as an AcMNPV bacmid deleted for *lef-2* was unable to produce infectious virus (40).

Ac7 (201aa:23.6kDa) (Orf 603). This gene is only found in three baculovirus genomes in addition to AcMNPV, including ClbiNPV, PlxyNPV and RoMNPV. Deletion from AcMNPV did not affect replication in cell culture or in *T. ni* larvae (44).

Ac8 (245aa:28.6kDa), (Bm1:245aa:28.8kDa) (polyhedrin, occlusion body protein). Homologs of polyhedrin are found in all baculovirus genomes, except for that of the dipteran virus (CuniNPV). Surprisingly, CuniNPV has an occlusion body protein unrelated and about three times as large as polyhedrin of other baculoviruses (45, 46). Because of unexpected patterns of phylogeny of AcMNPV polyhedrin, it has been suggested that it is a chimera derived from both Group I and II sequences (47). It is generally thought that polyhedrin serves to stabilize baculovirus virions in the environment allowing them to persist indefinitely. The polyhedrin gene is nonessential in cell culture, and occlusion-positive and negative plaques can be readily be distinguished. This, in combination with the strength of the polyhedrin promoter, led to the use of the polyhedrin locus as the site for the production of recombinant baculoviruses (4, 5). The crystal structure of AcMNPV polyhedra has been described (48).

Ac9 (543aa:60.7kDa), (Bm2:542aa:60.9kDa) (pp78/83, Orf1629). Homologs of pp78/83 are found in all lepidopteran NPV (I and II) genomes. It is an essential gene. Because it is located adjacent to the

polyhedrin gene, it was originally manipulated via complementation to elevate the frequency of obtaining recombinant baculoviruses at the polyhedrin locus (49). It is phosphorylated (50) and is a structural protein located at one end of nucleocapsids (50, 51). Ac9 is a Wiskott-Aldrich syndrome protein (WASP)-like protein involved in nuclear actin assembly during the baculovirus infection that leads to movement of virions through the cytoplasm of the infected cell (52, 53).

Ac10 (272aa:32kDa), (Bm3:275aa:32.4kDa) (Protein kinase-1 (PK-1)). Homologs of pk-1 are found in lepidopteran NPV and GV genomes, but not in other lineages. AcMNPV pk-1 shows significant relatedness to some insect orfs, e.g., *Anopheles gambiae* (E = 3e-18). Purified pk-1 was able to phosphorylate histone substrates (54). Ts mutants characterized for defects in very late gene expression contained mutations in *pk-1* (55). In addition, inactivation of pk-1 mRNA using DNAzyme technology caused a reduction in the expression from the polyhedrin promoter (56). PK-1 also may be associated with a very late transcription complex and be involved in the phosphorylation of LEF-8 (57). Purified PK1 of SpltNPV-I was found to bind to the polyhedrin promoter, suggesting that it might act as a very late gene transcription factor (58). It is likely an essential gene as an insertion mutant in the BmNPV homolog (Bm3) could not be isolated (15).

Ac11 (340aa:40.1kDa), (Bm4:340aa:39.8kDa). Homologs of Ac11 are found in all lepidopteran Group I NPV and in one Group II (LdMNPV) genomes, but not in other lineages. Ac11 is likely to be nonessential because when it was interrupted by insertional/deletion mutagenesis in BmNPV (Bm4), the virus appeared to be viable (15).

Ac12 (217aa:25.4kDa). Homologs of Ac12 are found in only three additional lepidopteran NPVs, including PlxyNPV, RoMNPV and LdMNPV. Analysis by HHpred (59) predicts that it contains an F-box domain that is involved in a ubiquitin pathway. It shows limited similarity to the tryptophan repeat gene family proteins of *Amsacta moorei* entomopoxvirus (E = 0.12) (see also Ac30).

Ac13 (327aa:38.7kDa), (Bm5:331aa:39.3kDa). Homologs of Ac13 are found in all Group I, II, and GV genomes, but not in other lineages. Analysis by HHpred (59) predicts that it contains coiled-coil regions and has some structural similarity to fibrinogen and borealin, a chromosomal passenger protein that is involved in the stability of mitotic spindles. Ac13 is likely to be nonessential because when it was interrupted by insertional/deletion mutagenesis in BmNPV (Bm5), the virus appeared to be viable (60). In addition Bm5 appears to encode a late expressed protein not associated with BV or ODV (61).

***Ac14 (266aa:30.8kDa), (Bm6:270aa:31.1kDa) (Lef-1, DNA primase).** LEF-1 is a core gene present in all baculovirus genomes. Purified LEF-1 has DNA primase activity (41). It interacts with Ac6 (LEF-2) (37), the baculovirus DNA primase accessory factor and is required for transient DNA replication (38, 39). Lef-1 is likely an essential gene as a deletion/insertion mutant in the BmNPV homolog (Bm6) could not be isolated (62).

Ac15 (506aa:57kDa), (Bm7:506aa:57.0kDa). Ac15 encodes ecdysteroid UDP-glucosyltransferase (63) and homologs are found in all Group I, II, and most GV genomes, but not in other lineages. Homologs are found in a variety of insects, e.g., *B. mori* (E = 3e-50). The function of EGT is to block molting and pupation in infected larvae by catalyzing the transfer of glucose from UDP-glucose to ecdysteroids, thereby inactivating these insect molting hormones (64, 65). This is thought to prolong the feeding stage of infected larvae, thereby allowing the virus to replicate over a longer period in larger larvae, resulting in a higher yield of virus. AcMNPV mutants in which the *egt* gene is inactivated are viable (65). Spontaneous deletions of the *egt* gene commonly occur in cell culture (66).

Ac16 (225aa:25.9kDa), (Bm8:229aa:26.2kDa) (BV/ODV-E26). Homologs of Ac16 are found in all lepidopteran Group I NPV genomes, but not in other lineages. Ac16 interacts with fp25 (Ac61), forms a complex with cellular actin (67) and is palmitoylated (68). It was found to be associated with the envelopes of both BV and ODV and was called BV/ODV-E26, and was also identified as being associated with ODV

by mass spectrometry analysis (36). However, in BmNPV, the homolog of AcMNPV Ac16 (Bm8) was not identified as a virion structural protein (69). It was suggested that the difference in results was due to the source of the antibody (70). A mutant, in which AcMNPV orf16 (called DA26) was insertionally inactivated was viable and showed no difference from wt in *T. ni* or *S. frugiperda* cells or larvae (71). In BmNPV, attempted isolation of null mutants of BmNPV (Bm8) was not successful, but a C-terminal deletion mutant was viable (69). However, this mutant was unable to produce BV titers as high as wt. In BmNPV, the Ac16 homolog (Bm8) directly interacts with IE-1 (72). Similar observations were made in AcMNPV with Ac16 interacting with both IE-1 and IE-0 (73). In AcMNPV, deletion of Ac16 resulted in a delay in full levels of DNA synthesis and BV production in one study (74) but had lesser effects in another (73). Analysis by HHpred (59) predicts that it contains a coiled-coil region.

Ac17 (209aa:23.9kDa), (Bm9:210aa:24.1kDa). Homologs of Ac17 are found in all Group I and II (except TnSNPV and ChchNPV) NPV genomes. It is an early expressed gene with a product of about 19 kDa that localizes to the cytoplasm (75). A homolog of Ac17 in HearNPV (He128) is expressed late and was found in the cytoplasm (76). Deletion of Ac17 did not affect DNA synthesis, although BV production was reduced by up to a factor of 10 to 100 (74). Deletion of both Ac16 and Ac17 was similar to the Ac17 deletion although there were more significant delays for DNA replication and BV titers to reach levels similar to the Ac17 deletion alone (74). Similar results were reported for a BmNPV bacmid knock out. In addition, the mutant BmNPV took significantly longer to kill larvae and required higher titers BV to achieve an LD_{50} (77). The predicted size of Ac17 is longer than previously reported (12).

Ac18 (353aa:40.9kDa), (Bm10:356aa:41.5kDa). Homologs of Ac18 are found in all Group I and II NPV genomes. An AcMNPV bacmid deleted for Ac18 was infectious with an LD50 in *T. ni* larvae similar to wt, but took somewhat longer to kill larvae than wt (78).

Ac19 (108aa:12.2kDa), (Bm11:110aa:12.5kDa). Homologs of Ac19 are found in all Group I and most group II NPVs. Ac19 is likely to be nonessential because when it was interrupted by insertional mutagenesis in BmNPV (Bm11), the virus appeared to be viable (15).

Ac20/21 (417aa:47.7kDa), (Bm12:440aa:50.0kDa) (arif-1). This region has been resequenced in AcMNPV strains C6 and also in strains E, E2, and L1, and in all cases it was found that Ac20 and 21 are a single orf (11), see also (12). In addition, these two orfs are combined as a single orf in several closely related viruses. Homologs of arif-1 are present in all Group I and most Group II genomes. AcMNPV orf20/21 is called actin-rearrangement-inducing factors (arif-1). It is expressed as an early gene and transfection of a plasmid containing this gene into Tn-368 cells is able to induce actin rearrangement (79). Arif-1 was found to colocalize with F-actin at the plasma membrane and deletion mutants showed a loss of actin concentration at the plasma membrane. Deletions of the C-terminal half of the gene or insertion of the LacZ gene near the center of the orf resulted in constructs that showed no significant loss of infectivity in Tn-368 or Sf cells (11). Analysis by HHpred (59) predicts that Ac21 has an extensive region that resembles structures found in the largest subunit of RNA polymerase II, fibrinogen, and rhodopsin.

***Ac22 (382aa:43.8kDa), (Bm13:382aa:43.8kDa) (PIF-2).** This gene is a member of the per os infectivity factor (80) gene group. These genes can be deleted and the mutant are still infectious for cultured cells, but are not orally infectious for insects (81). It is present in all baculovirus genomes and is also present in a nudivirus genome (82). For more information see Ac138 (p74).

Ac23 (690aa:79.9kDa), (Bm14:673aa:78.0kDa), F protein. Ac23 is a homolog of the predicted fusion protein (F) of Group II lepidopteran NPVs, GVs, and the dipteran virus (CuniNPV). Homologs are found in all baculoviruses except the hymenopteran viruses. In Group II viruses such as LdMNPV, Ac23 homologs (*ld130*) encode low pH-activated fusion proteins. In Group I viruses, they appear to be inactive as fusion proteins and have been replaced by *gp64*, which is not found in the Group II viruses, GVs, or hymenopteran or dipteran viruses. Homologs of the F gene are also found as the env gene of insect retroviruses (83), and are also present in some insect genomes (84). Although inactive in Group I viruses,

the Acorf23 homolog is glycosylated and associated with the envelope of BV and with the membranes of OpMNPV-infected cells (85). In AcMNPV, Ac23 is also associated with BV membranes and its deletion from the genome results in infectious virus, but the time to kill larvae was somewhat extended (10). Ac23 was found to be associated with ODV as was the homolog in CuniNPV (36, 86).

Ac24 (169aa:19.2kDa), (Bm15:169aa:19.4kDa), Protein kinase interacting protein (PKIP). Homologs are found in all Group I and II NPV genomes. This gene product was found to interact with AcMNPV protein kinase I (see Ac10) in a yeast two-hybrid assay. It stimulates PK-1 activity in vitro. PKIP appears to be essential as attempts to isolate a deletion mutant were unsuccessful (87). Ts mutants in pkip are defective in very late transcription (88). BmNPV insertion mutations of this gene (Bm15) also could not be isolated (15).

Ac25 (316aa:36.6kDa), (Bm16:317aa:36.7kDa), DBP. Ac25 encodes a single-stranded DNA binding protein called DBP. Homologs are found in all sequenced baculovirus genomes, except that of the dipteran (CuniNPV) (although an orf is present with ~50% identity over 22 aa) and in some instances multiple copies of the *dbp* gene are present. It has properties similar to the other baculovirus SSB, LEF-3 (Ac67), in that it interacts with itself and is capable of both unwinding and annealing DNA (89). It is an essential gene, as bacmids lacking Ac25 are noninfectious and appear to produce defective nucleocapsids. Although not a virion structural protein, DBP exhibits a tight association with subnuclear structures, suggesting that it is a component of the virogenic stroma (89), and when DBP was deleted from an AcMNPV bacmid, cells transfected with this construct appeared to lack a virogenic stroma. This suggested that *dbp* is required for the production of nucleocapsids and the virogenic stroma (90).

Ac26 (129aa:14.6kDa), (Bm17:129aa:14.5kDa). Homologs of Ac26 are found in the genomes all Group I, and most Group II NPV genomes. Ac26 is likely to be nonessential because when it was interrupted by insertional mutagenesis in BmNPV (Bm17), the virus appeared to be normal (15).

Ac27 (286aa:33.3kDa), (Bm18:292aa:34.0kDa), iap-1. Ac27 is a member of the inhibitor of apoptosis (*iap*) gene family. Up to 5 *iap* homologs are found in baculovirus genomes (17), however AcMNPV has two copies, *iap-1* and *-2*. Unlike *iap-2*, which is found in both Group I and II NPVs, *iap-1* appears to be confined to Group I lepidopteran NPVs and is a member of a lineage distinct from the other iap homologs. Deletion mutants of *iap-1* were similar to wt in their replication in cells lines and larvae of *T. ni* and *S. frugiperda*. However, when they were co-infected with wt virus in Tn-368 (but not Sf-21) cells, the mutants appeared to out compete wt virus (91). Evidence suggested that transfection of AcMNPV iap-1 into T. ni cells suppressed apoptosis by HearNPV infections, although a recombinant HearNPV expressing iap-1 also suppressed apoptosis, BV production was not rescued (92). In another investigation in EppoMNPV, The iap-1 gene was able to delay apoptosis onset caused by inducing agents such as actinomycin but was not able to prevent apoptosis upon prolonged exposure of the cells to the inducer (93).

Ac28 (173aa:20.4kDa), (Bm19:173aa:20.3kDa), Lef-6. Homologs of lef-6 are found in the genomes of all lepidopteran NPVs and GVs. It was originally identified because it was required for transient transcription of late genes (94). A bacmid deleted for lef-6 was infectious, but the virus was severely compromised. The major effect appeared to be reflected in a delay in the onset of late transcription (95). Using the HHpred program (59), AcMNPV LEF-6 showed a 68% probability of being related to the RNA binding domain of the mRNA export factor TAP (96).

Ac29 (71aa:8.6kDa), (Bm20:71aa:8.6kDa). Homologs of Ac29 are found in all Group I and most Group II and GV genomes. It may be an essential gene as a deletion/insertion mutant in the BmNPV homolog (Bm20) could not be isolated (15).

Ac30 (463aa:54.7kDa), (Bm21:472aa:55.8kDa). Homologs of Ac30 are present in the genomes of all Group 1 NPVs. It has homology to a family of genes that encode tryptophan repeat gene family proteins (see also Ac12) that are also found in entomopox viruses. e.g., *Melanoplus sanguinipes* entomopoxvirus

(E = 1e-11). These proteins contain 3 to 12 copies of a 23-amino acid sequence containing tryptophan, leucine and isoleucine residues (97). Ac30 is likely to be nonessential because when the ortholog (Bm21) was interrupted by insertional/deletion mutagenesis in BmNPV, the virus appeared to be normal, although it resulted in a longer survival time suggesting that the mutant was less virulent that wt (98).

Ac31 (151aa:16.2kDa), (Bm23:151aa:16.3kDa), SOD. Ac31 has homology to super oxide dismutase (59). Homologs are found in the genomes of almost all lepidopteran baculovirus genomes (it appears to be absent only in EppoNPV, a member of Group II, and SpliGV). It has a high degree of similarity to SOD from a variety of insects, including *B. mori* (E = 8E-49). Insect hemocytes are phagocytic cells similar to neutrophils and can destroy invading pathogens by the production of superoxide (99). Superoxide can be inactivated by SOD by converting it to hydrogen peroxide, which is also toxic, but can itself be inactivated with catalase yielding water and O_2. Many baculoviruses may infect hemocytes and in this manner can spread an infection throughout an insect. The expression of viral SOD might mitigate the effects of superoxide production by hemocytes. An enzymatic activity could not be confirmed for AcMNPV SOD and AcMNPV deleted for *sod* replicated normally in cultured cells and insect larvae. The *sod*-deleted viruses showed no reduction in replication when grown in the presence of paraquat, a superoxide anion inducer (100). However, deletion of the *sod* gene from BmNPV (Bm23) indicated that it was essential for replication of that virus in BmN cells (101).

Ac32 (181aa:20.6kDa), (Bm24:182aa:20.8kDa), fgf. Ac32 has homology to fibroblast growth factor (FGF). Homologs are found in the genomes of all lepidopteran baculoviruses and may reflect several independent lineages. AcMNPV *fgf* is most closely related to a non-baculovirus gene in *D. melanogaster* called *branchless* (E = 3e-24). In contrast, a Group II *fgf* homolog from LdMNPV is less closely related to the insect homologs (e.g. *D. melanogaster* branchless E = 2e-10) and the GV homologs show only limited similarity to NPV *fgf* homologs (e.g., AcMNPV vs CpGV FGFs = ~10% identity). It has been suggested all of the NPV *fgf*s are monophyletic, however the possibility of at least two capture events of fgf in the GVs has not been ruled out (102). AcMNPV FGF is a secreted protein that stimulates insect cell motility (103). In BmNPV, the fgf homolog is glycosylated, which is essential for its (104, 105) and binds to an insect receptor of FGF/*branchless* called *breathless* (104). Although the deletion of *fgf* in AcMNPV showed no differences from wt on cultured cells (106), the time of death was delayed when fed to two insect species (107). Similar results were observed for a BmNPV *fgf* deletion (108). It has been suggested that FGF may play a role in dissemination of the virus within the host insect (107). Recent evidence suggests that vFGF initiates a cascade of events that may accelerate the establishment of systemic infections. This involves two processes. vFGF from virus infected midgut cells diffuse through the basal lamina and attract tracheal cells so that they are adjacent to infected midgut cells but separated by the basal lamina. vFGF then activates FGF receptors located on the tips of tracheal cells. This leads to the activation of matrix metalloproteases located in the same subcellular region via a MAP kinase or NFkB pathway. Matrix metalloproteases subsequently activate effector caspases that move extracellularly so that they are positioned for the degradation of the basal lamina by digestion of the laminin component. The delaminated tracheal cells are then susceptible to virus infection. This allows the transit of the virus through tracheal cells to other tissues and results in the systemic infection (109). This theory is supported by evidence for the activation of matrix metalloproteinases, the activation of effector caspases, and the degradation of laminin after the per os infection of midgut cells.

Ac33 (182aa:20.8kDa) (polynucleotide kinase (PNK)). Homologs of Ac33 are found in most Group II and a few Group I and GV genomes. However, homology searches indicate that it has significant similarity to polynucleotide kinase-3'-phosphatase of *Apis mellifera* (E = 9e-36) and somewhat lesser to the human homolog (E = 1e-28). In other organisms this enzyme has functions similar to T4 PNK. It is predicted to have structural similarity to Chain B of a DNA repair enzyme, polynucleotide kinase with an E value of $1.0e^{-32}$ (110). As previously suggested, Ac86 also has structural similarity to histidinol-phosphatase (an enzyme in the histidine biosynthesis pathway) (111). A homolog is not present in BmNPV (16).

Ac34 (215aa:24.9kDa), (Bm25:215aa:24.8kDa). Homologs of Ac34 are found in Group I and II NPV genomes. It may be an essential gene as an insertion mutant in the BmNPV homolog (Bm25) could not be isolated (15).

Ac35 (77aa:8.7kDa), (Bm26:77aa:8.7kDa), ubiquitin-like protein. Homologs of ubiquitin are found in all lepidopteran Group I NPVs and GVs and most Group II NPVs, but are not present in hymenopteran or dipteran baculoviruses. Ac35 is expressed from both early and late promoter elements (112, 113). The phylogenetic tree indicates that, whereas the ubiquitin of most eukaryotes is almost invariant, the baculovirus tree shows a higher degree of phylogenetic diversity, particularly between GVs and NPVs, suggesting that it may have been independently incorporated into a viral genome more than once. It is highly conserved among different NPVs showing at least 76% amino acid sequence identity. The most closely related non-baculovirus homologs of Ac35 are from vertebrates, e.g., rat (E = 2e-28). It is BV associated (26) and appears to be present on the inner surface of viral envelopes (114). A viral mutant with a frameshift of Ac35 is viable, but a 5–10-fold reduction in BV was observed (115). It has been suggested that viral ubiquitin may inhibit steps in the host degradative pathway to stabilize what would otherwise be a short-lived viral protein (116). The *Spodoptera litura* NPV genome was found to contain a gene that is a fusion of *ubiquitin* and *gp37* (=Ac64). In addition, it was noted that unfused homologs of both these proteins are found in entomopox viruses (117). Although the significance of the linkage of these two proteins is not known, other such proteins have been termed 'rosetta stone' proteins because they reveal proteins that interact with one another and participate in the same molecular pathways. Consequently, when a mutation event occurs that leads to the fusion of two proteins that normally function together, the mutation is preserved because such a linkage is a normal feature of the two proteins. The presence of homologs of both these proteins in two disparate families of viruses along with the presence of a fused orf in the SlNPV genome, suggests that these orfs may participate in the same pathway, possibly as participants in a ubiquitin pathway or in ubiquitin inhibition.

Ac36 (275aa:31.3kDa), (Bm27:277aa:31.5kDa), 39K;pp31. Pp31 was originally identified because it contains an early promoter that is stimulated by IE-1 (118). Homologs are found in all lepidopteran NPV and GV genomes. It is phosphorylated and localized to the virogenic stroma of infected cells, and is capable of binding to DNA but is not a virion structural protein (119), although it was reported to be BV associated in a proteomic study (26). Purified PP31 was found to bind to single-stranded and double-stranded DNA with equal affinities and inhibited transcription in vitro (120). Phosphorylation of pp31 appeared to be a dynamic process (121). Several basic regions were identified that may be involved in nuclear localization or DNA binding (122). Pp31 stimulates late gene expression in a transient transcription assay (123). Deletion of the *pp31* homolog in BmNPV (Bm27) resulted in virus that, although viable, showed a reduction in late gene transcription, a 100 fold reduction in BV production, and improper formation of the virogenic stroma (62). Similar results were obtained for an AcMNPV bacmid deleted for *pp31* and it was observed that the deletion resulted in a significant decrease of the transcription of six late genes (124).

Ac 37 (112aa:13.1kDa), (Bm28:112aa:13.1kDa), Lef-11. Lef-11 is present in all baculovirus genomes, except the dipteran CuniNPV. It was identified as being stimulatory for late gene expression in a transient transcription assay (39). An AcMNPV bacmid deleted for *lef-11* failed to replicate and no DNA synthesis or late gene transcription were evident, indicating that it is an essential gene. Although LEF-11 localizes to nuclei of infected cells and appears to be essential for DNA replication, no domains associated with other factors involved in DNA replication have been reported and its role in DNA replication is not known (95).

Ac38 (216aa:25.3kDa), (Bm29:217aa:25.5kDa), Nudix, ADP-ribose pyrophosphatase (ADPRase). Homologs of Ac38 are found in all lepidopteran NPVs and GVs. It contains a conserved Nudix (nucleotide diphosphate X) motif ($GX_5EX_7REUXEEXGU$; X= any aa, U represents I, L, or V) (125) and has a homology to ADPRase, a subfamily of Nudix pyrophosphatases. Ac38 was shown to have ADPRase activity and a deletion mutant was severely compromised and produced BV at 1% the level of wt (126).

Proteins of the nudix superfamily are common in all organisms and have been reported in other viruses including T4 bacteriophage, African swine fever virus (ASFV), and poxviruses. A vaccinia virus nudix protein may negatively regulate viral gene expression by acting as a decapping enzyme (127). Deletion of the gene in vaccinia resulted in smaller plaque and low virus yield (128), similar to the *Ac38*-deleted AcMNPV. Ha33 of the *Heliocoverpa armigera* NPV is a homolog Ac38 and was found to be associated with the envelope of budded virions (129).

Ac39 (363aa:43.5kDa), (Bm30:362aa:43.4kDa). Homologs of Ac39 are found in a few Group I NPV closely related to AcMNPV and two Group II (Adho-and ClbiNPV) genomes. Deletion showed no effects on growth curves or virus production (130).

***Ac40 (401aa:47.5kDa), (Bm31:399aa:47.3kDa) P47, a subunit of the baculovirus polymerase.** P47 homologs are found in all baculovirus genomes. P47 was originally identified as the site of a ts mutation that caused a defect in late gene expression (131, 132). Structural predictions suggest that it is related to the RNA polymerase alpha subunit (see Chapter 6). P47 was found to be required for transient late gene transcription (123) and to be a component of the baculovirus late polymerase complex (133). It is likely an essential gene as a deletion/insertion mutant in the BmNPV *lef-8* homolog (Bm31) could not be isolated (62).

Ac41 (181aa:21.1kDa), (Bm32:183aa:21.1kDa), Lef-12. Lef-12 is found in about one-half the Group I and Group II NPV genomes sequenced. Although 18 genes were originally identified as being involved in transient expression from a late promoter (123), when individually cloned, the genes failed to support late transcription. Because of its close proximity to Ac 40 (p47), Ac41 (lef-12) had not been identified in the initial screen. It was subsequently demonstrated to be required for transient late gene transcription in *S. frugiperda* cells (134, 135), but not in *T. ni* cells (134). Mutants with *lef-12* interrupted by insertional mutagenesis or by mutation of the ATG translation initiation codon were viable in both *S. frugiperda* and *T. ni* cells, although reduced yields of BV were observed (20-40% or wt) in both cell lines and the infection cycle appeared to be slowed (136). Although expressed as an (aphidicolin sensitive) late gene, initiation of *lef-12* mRNA did not appear to occur at conventional late (or early) promoter elements. It was suggested that *lef-12* may be functionally redundant in the AcMNPV genome and, therefore, it is not essential for late transcription when the rest of the virus genome is present (136).

Ac42 (506aa:59.1kDa), (Bm33:506aa:59.2kDa), gta. Ac42 has homology to 'global transactivator,' the DEAD-like helicase superfamily that are enzymes involved in ATP-dependent unwinding of DNA or RNA. They contain an SNF2 family N-terminal domain that is present in proteins involved in some processes, such as regulation of transcription, DNA recombination and repair, chromatin unwinding, and other functions. Homologs of this gene are found in all Group I NPV genomes. It has significant similarity to a wide variety of orfs from bacteria to marsupials, e.g., (E = 9e-61). Deletion of the Ac43 homolog from BmNPV (Bm33) did not cause any defects in BV or ODV production in BmN cells. Assays in *B. mori* larvae showed that the mutant, although similar in infectivity to the wt, took about 15 hr longer to kill when administered either by injection or per os (137).

Ac43 (77aa:8.8kDa), (Bm34:78aa:9.0kDa). Homologs of Ac43 are found in all Group I and most Group II NPV genomes. It appears to be involved in late and very late gene expression as deletion of BmNPV Bm34 resulted in a reduction in occlusion body production and a lengthening of the time to death in larvae. These effects were attributed to a down regulation of *vlf-1* which is required for very late gene expression along with the reduction expression of the *fp25k* (=Ac61) gene (138).

Ac44 (131aa:15kDa), (Bm35:131aa:15.0kDa). Homologs of Ac44 are found in all of Group I and one Group II (SeMNPV) lepidopteran NPVs. Ac44 is 131 amino acids and shows homology to a 64.2 kDa inhibitor of apoptosis/RING-finger protein in the genome of *Spodoptera frugiperda* ascovirus 1a (E = 1e-04). The SeMNPV homolog is more closely related to the ascovirus 1a orf (E = 2e-55) than to its Group I baculovirus homologs. Ac44 is likely to be nonessential because when it was interrupted by insertional

mutagenesis in BmNPV (Bm35), the virus appeared to be normal (15).

Ac45 (192aa:22.7kDa), (Bm36:193aa:22.5kDa). Homologs of Ac45 are found in four close relatives of AcMNPV (PlxyNPV, BmNPV, RoMNPV, and MaviMNPV). Sequences located within Ac45 appeared to be required for Ac41 expression in a transient late transcription assay (135). Ac45 is likely to be nonessential because when the ortholog was interrupted by insertional/deletion mutagenesis in BmNPV (Bm36), the virus appeared to be normal (15).

Ac46 (704aa:79.1kDa). (Bm37:702aa:79.2kDa), ODV-E66. Ac46 is a component of ODV envelopes (139). Homologs of this gene are found in the genomes of all Group I NPVs, GVs, and most Group II NPVs, but not in hymenopteran or dipteran viruses. Two copies of the gene are present in some genomes (e.g., SeMNPV). When the N-terminal 23 amino acids of ODV-E66 are fused to a reporter gene, it is targeted to the nucleus (140). Ac46 shows 100% probability of being related to hyaluronidase of *Streptococcus pneumonia* using the HHpred program (59) and has been shown to have hyaluronan lyase activity that is capable of digesting hyaluronan, a polysaccharide that is a major component of the extracellular matrix (141). The extracellular matrix is an extracellular component of tissues that provides structural support for cells. When the homolog of ODV-E66 was inactivated in BmNPV (Bm37), the mutant, although viable, took more time to kill insect larvae (60).

Ac47 (88aa:10.5kDa), (Bm38:89aa:10.5kDa), TRAX-like. Ac 47 homologs are found in 5 Group I lepidopteran NPVs closely related to AcMNPV. It shows 27% identity to homologs of a protein called translin-associated factor X (Trax). Although TRAX interacts with translin, which may be involved in responses to DNA damage, transport of RNA, and control of translation, its function is not known (142). Ac47 is likely to be nonessential because when the ortholog was interrupted by insertional mutagenesis in BmNPV (Bm38), the virus appeared to be normal (15). This gene was referred to as ETS and transcriptional data for Ac47 in relation to PCNA has been described (143).

Ac48 (113aa:12.9kDa). Ac48 homologs are found in the genomes of 10 Group I lepidopteran NPVs. A homolog of Ac48 is not present in the BmNPV genome (16). This gene was referred to as ETM (the mid-sized orf in the EcoRI T fragment) and transcriptional data for Ac48 in relation to PCNA has been described (143).

Ac49 (285aa:32.1kDa) (PCNA). Ac 49 has homology to proliferating cell nuclear antigen (PCNA). PCNA homologs have been found in the genomes of four Group I and two Group II lepidopteran NPVs. The Group I PCNA homologs appear to be insect-derived and show a high degree of similarity to insect PCNAs, e.g., *S. frugiperda* (E = 6e-65). In contrast, the PCNA homologs of the two Group II viruses (TnSNPV and ChchNPV) belong to a different lineage and do not show such a close relationship to insect PCNAs, e.g., *S. frugiperda* (E = 7e-20) and are even more distantly related to the Group I baculovirus PCNAs than to those of insects. Although eukaryotic PCNA lacks an enzymatic function, it plays a role in DNA synthesis, DNA repair, and cell cycle progression. It functions as a sliding circular clamp that mediates protein interactions with DNA and is required for the coordinated synthesis of both leading and lagging strands at the replication fork during DNA replication (144). In AcMNPV it is not an essential gene (143, 145, 146) and did not appear to elevate DNA replication in transient replication assays (38). A homolog of PCNA is not present in the genome of BmNPV (16).

***Ac50 (876aa:101.8kDa), (Bm39:877aa:101.8kDa), Lef-8, baculovirus RNA polymerase subunit.** Lef-8 was originally identified as a gene required for transient late gene expression (147). Homologs are found in all baculoviruses and are also in nudiviruses. LEF-8 contains a conserved motif found in other RNA polymerases and it is thought that this is part of the catalytic site (147, 148). It is a component of the baculovirus late RNA polymerase complex (133, 149). It is likely an essential gene; in BmNPV a ts mutant located in lef-8 was defective for BV production at the non permissive temperature (150). In addition, a deletion/insertion mutant in the BmNPV lef-8 homolog (Bm39) could not be isolated (62).

Ac51 (318aa:37.5kDa), (Bm40:319aa:37.8kDa), DnaJ domain protein. Homologs of Ac51 are found in all Group I and II lepidopteran NPV genomes. It is a DNA J domain protein and shows homology to a variety of bacterial proteins. In *E. coli*, DnaJ has been demonstrated to have chaperone activity and aids in folding of other proteins (151). The homolog of Ac51 in HearNPV (ha39) has an RNA recognition motif, localizes to the cytoplasm and is associated with BV (152, 26). It may be an essential gene as an insertion mutant in the BmNPV homolog (Bm40) could not be isolated (15).

Ac52 (194aa:123.2kDa), (Bm41:194aa:23.3kDa). Homologs of Ac52 are found in about one-half the Group I and II lepidopteran NPV genomes. Deletion of Bm41 resulted in reduction in BV production by 1000-fold and appeared to disrupt normal nucleocapsid envelopment and polyhedron formation in infected nuclei and resulted in a 14-fold elevation of LD_{50} in larvae and an increase in time to death (153). The predicted size of Ac17 is longer than previously reported (12).

Ac53 (139aa:17kDa), (Bm42:139aa:16.9kDa). Homologs of Ac53 are found in all baculoviruses, except the dipteran CuniNPV. Deletion of Ac53 indicated that it was an essential gene. The mutant bacmid was able to replicate DNA, but the virions were defective and appear to lack the nucleoprotein core (154).

Ac53a (78aa:8.6kDa), (Bm42a:78aa:8.6kDa), Lef-10. This orf was named Ac53a because it was not identified in the original AcMNPV genome sequence because it is a small orf encoding 78 aa and about half the 3' coding region overlaps the 5' region of Ac54. Homologs of lef-10 are found in the genomes of all Group I and most Group II NPV and GV genomes. Lef-10 was originally identified because it was required for late gene expression (14). It is likely an essential gene, as an insertion mutant in the BmNPV homolog (Bm42a) could not be isolated (62).

***Ac54 (365aa:42.1kDa), (Bm43:365aa:42.0kDa), Capsid protein, (vp1054).** Homologs are found in all baculovirus genomes. Ac54 encodes a protein required for nucleocapsid assembly. A ts mutant failed to produce nucleocapsids at the nonpermissive temperature, indicating that it is an essential gene. It is found associated with both BV and ODV (155). It interacts with 38K (Ac98) (156). An insertion mutant of the BmNPV homolog could not be isolated (15).

Ac55 (73aa:8.2kDa), (Bm44:77aa:8.6kDa). Homologs of Ac55 are found in the genomes of all Group I and most of the Group II NPVs. It is likely to be nonessential because when the ortholog was interrupted by insertional/deletion mutagenesis in BmNPV (Bm44), the virus appeared to be normal (15).

Ac56 (84aa:9.9kDa), (Bm45:84aa:9.9kDa). Homologs of this orf are found in genomes of all Group I and most Group II NPVs. It is likely to be nonessential because when it was interrupted by insertional/deletion mutagenesis in BmNPV (Bm44), the virus appeared to be normal (15).

Ac57 (161aa:19kDa), (Bm46:161aa:20.2kDa). Homologs of this orf are found in the genomes of all but one Group I (MaviMNPV) and one Group II (AdhoNPV) NPV. It is likely to be nonessential because when it was interrupted by insertional/deletion mutagenesis in BmNPV (Bm46), the virus appeared to be normal (15).

Ac58/59 (172aa:20.3kDa), (Bm47:171aa:37.8kDa), ChaB-like. These two orfs are likely a single gene, as homologs are fused in other baculoviruses and they were also found to be joined when the region was resequenced in the C-6 strain (12). This results in an orf predicted to encode 172 amino acids. Homologs of this orf are found in the genomes of all Group I and all but one (LeseNPV) Group II NPVs. It has a ChaB domain. In *E. coli*, ChaB is thought to regulate ChaA, a cation transporter protein. It was found to localize to nuclei of infected cells (157) and was associated with AcMNPV ODV (36). It is also BV associated (26). It is likely to be nonessential because when it was interrupted by insertional mutagenesis in BmNPV (Bm47), the virus appeared to be normal although the motility of infected larvae may have been limited (60).

Ac60 (87aa:10.1kDa), Bm48:83aa:9.7kDa) (ChaB-like). Homologs of Ac60 are found in the genomes of

all Group I and II NPVs and most GVs. Similar to Ac58/59, Ac60 also has a ChaB domain. It is surprising that Ac58/59 and Ac60 are both predicted to encode ChaB domains as they do not show much sequence similarity. Alignment of the sequences resulted in an amino acid sequence identity of 15%; however, it required the insertion of several gaps, so the significant of the relatedness is not clear. In SpliNPV, two adjacent ChaB homologs were also identified. Evidence suggests that they may be DNA binding proteins (158). Ac60 may be an essential gene as an insertion/deletion mutant in the BmNPV homolog (Bm48) could not be isolated (15).

Ac61 (214aa:25.2kDa), (Bm49:214aa:25.3kDa), Few polyhedra (fp, fp-25). Homologs of the *fp* gene are found in the genomes of all lepidopteran NPVs and GVs. AC61 has been shown to be BV associated (26). Deletion is not lethal, but results in a 'few polyhedra phenotype' (159, 160). FP mutants are defective in virion occlusion and nucleocapsid envelopment in nuclei and release two- to fivefold more infectious BV than wt in infected Sf9 cells (160, 161). The nonlethal, but readily distinguishable phenotype has facilitated investigations on this gene. FP mutations often result from the insertion of host DNA into the fp gene (159, 162). They can also be the result of errors in DNA replication (163). Mutations in the *fp* gene result in a reduction in polyhedrin gene (but not *p10*) transcription (160). Mutations also appear to affect the levels and nuclear transport of Ac46 (ODV-E66), an ODV envelope protein (164, 165). In BmNPV *fp* mutants, the few virions that were occluded appear to lack envelopes (166). A combination of reduction in the level of polyhedrin and an ODV envelope protein could contribute to the FP phenotype. The defect in occlusion and in the ODV envelope could lead to the availability of more virions for budding. A reduced level of liquefaction of larvae was also noted with an fp mutant in BmNPV (166). This was attributed to the involvement of Bm49 in the regulation of v-cathepsin expression (167). In *T. ni* cells, double *p35* (see Ac135) and *fp* mutants underwent apoptosis, whereas p35 mutants alone did not, suggesting that the *fp* gene may have pro-apoptotic properties in this cell line (168). In contrast, in BmNPV infected BmN cells, deletion of fp25k did not affect apoptosis induced by a virus with p35 deleted (169).

***Ac62 (516aa:59.3kDa), (Bm50:490aa:56.4kDa), Lef-9, baculovirus RNA polymerase subunit.** Lef-9 homologs are present in all baculovirus and also nudivirus genomes. It was found to be required for transient late gene expression (14) and subsequently shown to be a subunit of the baculovirus RNA polymerase (133). It contains a 7-amino acid motif (NTDCDGD or NRDCDGD except NADFDGD in the dipteran virus) similar to the Mg++ binding sequence (NADFDGD) found in the catalytic center in large RNA polymerase subunits of a few DNA-dependent RNA polymerases (14). The D residues bind Mg++ and are conserved in all these sequences. It is likely an essential gene as an insertion/deletion mutant in the BmNPV homolog (Bm50) could not be isolated (62).

Ac63 (155aa:18.5kDa), (Bm51:155aa:18.5kDa). Homologs of Ac63 are found in five Group I and six Group II lepidopteran NPVs. A homolog in a nudivirus has been reported (170). It appears to be associated with BV envelopes (171). It is likely to be nonessential because when it was interrupted by insertional/deletion mutagenesis in BmNPV (Bm51), the virus appeared to be normal (15).

Ac64 (302aa:34.8kDa), (Bm52:294aa:33.8kDa) (GP37/P34.8, spindlin, fusolin, spheroidin-like protein). The terminology of Ac64 has a confusing history and in addition to gp37 has been referred to as p34.8, spindlin, fusolin, or spheroidin-like protein because of homology with an entomopox virus gene (172, 173). Evidence suggests that it is not a spheroidin-like homolog (174). Homologs of gp37 have been found in the genomes of most Group I (all but AgMNPV) and II (all but AdhoNPV) NPVs and three GVs (HaGV, XeniGV, and CpGV) and in AcMNPV it is expressed as a late gene (175). It is related to orfs in entomopox viruses and a variety of eubacteria, e.g., *Vibrio alginolyticus* (E = 5e-27). A homolog of Ac64 is referred to as chitinase B in the marine bacterium *Pseudoalteromonas* sp. and was found to bind to, but not digest chitin (176). The gp37 homolog in SpltNPV has been reported to contain chitin binding domains and is capable of binding to chitin (177). Insect proteins, such as the coagulation protein hemolectin, also have chitin-binding domains (178). Whether gp37 somehow inhibits or redirects such pathways remains to be determined. GP37 was reported to be polyhedron associated in AcMNPV and to be N-glycosylated

(173). It was also found to be BV associated (26). In OpMNPV infected *L. dispar* cells, gp37 was found to be an N-glycosylated protein located in cytoplasmic occlusions late in infection (179). The *gp37* gene is nonessential for replication in cell culture or *T. ni* larvae (172). Similar results were observed for the BmNPV homolog (Bm52) (15). The *Spodoptera litura* NPV genome was found to contain a gene that is a fusion of ubiquitin and gp37 (117) (for discussion, see *Ac35, ubiquitin*).

***Ac65 (984aa:114.3kDa), (Bm53:986aa:114.4kDa) (DNA polymerase).** Homologs are found in all baculoviruses. The nonbaculovirus homologs showing the highest level of similarity are found in herpesviruses, e.g., human herpes virus 7 (E = 1e-25), several protozoans, and archae. A DNA polymerase homolog was originally identified in the AcMNPV genome by hybridization with degenerate primers designed based on a highly conserved domain in other DNA polymerases (180). A 3'→5' exonuclease activity specific for single-stranded DNA was shown to be associated with the DNA polymerase from *Bombyx mori* NPV (BmNPV) (181) and AcMNPV (182), suggesting that a proofreading activity was associated with this enzyme. A purified DNA polymerase from AcMNPV was characterized as being active on singly primed M13 templates (183). The polymerase is highly processive on poly (dA)-oligo (182, 184). It is an essential gene because deletion is lethal in AcMNPV (185) and BmNPV (62).

***Ac66 (808aa:94kDa), (Bm54:805aa:93.3kDa).** Homologs of *Ac66* appear to be present in all baculoviruses. Many viruses appear to have two copies Ac66 of homologs, and some may have three copies (17). Ac66 is closely related to a variety of proteins including a predicted orf in the protozoan *Trichomonas vaginalis* (E = 6e-20), an actin binding protein in *Dictyostelium discoideum* (E = 1e-14), rabbit myosin heavy chain (E = 2e-14), and centromere protein E of *Canis familiaris* (E = 2e-14). It has a few conserved domains, including Smc, which is a chromosome segregation ATPase involved in cell division and chromosome partitioning, desmoplakin, the main adhesive junction protein in epithelia and cardiac muscle, and a domain in a chromosome segregation protein. It was found to be associated with AcMNPV (36) and HearNPV (Ha66) (186) ODV and AcMNPV BV (26). Ac66 is oriented in the opposite direction of DNA polymerase (Ac65) and its promoter region overlaps with the 5' region of the DNA pol orf. This orientation is conserved in many, if not all baculoviruses. Although the orfs adjacent to DNA polymerase in *Neodiprion* (e.g., NeSeNPV) and CuniNPVs show little homology to Ac66, they and Ac66 show homology to the same proteins, i.e., they both show almost 100% probability of being related to colicin 1a and myosin using the HHpred program (59). Consequently, it is likely that Ac66 is conserved throughout the baculoviruses. Ac66 is transcribed as a late gene and its expression does not affect the expression of DNA pol (187). An AcMNPV bacmid deleted for Ac66 was severely compromised and BV titers derived from transfected cells were reduced by over 99% compared with wt. In addition, at low titers the mutant BV appeared to infect single cells and was unable to spread to other cells. Although the nucleocapsids appeared to be normal and had an electron dense core, suggesting that they contained DNA, they appeared to be trapped in the virogenic stroma, suggesting that Ac66 was required for the efficient egress of virions from nuclei. The deletion did not affect the levels of DNA replication or polyhedrin transcription, but the production of occlusion bodies was eliminated (188). This suggests that Ac66 is required both for egress of virions from nuclei and also may be involved in the ennucleation of polyhedra. For additional discussion, see Chapter 5.

Ac67 (385aa:44.6kDa), (Bm55:385aa:44.9kDa), Lef-3. Lef-3 was originally found to be essential for DNA replication in transient assays (38, 39). Homologs of *lef-3* are found in the genomes of lepidopteran NPVs and GVs, but not those of hymenopteran or the dipteran NPVs. LEF-3 is a single-stranded DNA binding protein (SSB) (189). It also binds to helicase (190) and facilitates its transport into the nucleus (191). It can also drive nuclear transport into mammalian cells (192). It also may have a function in DNA replication in addition to its requirement as a helicase transport factor (193, 194). LEF-3 also interacts with alkaline nuclease and may regulate the function of this enzyme (195, 196); it is capable of both unwinding and annealing DNA depending on its concentration or redox state (197, 198); and it can facilitate the production of structures resembling recombination intermediates via strand exchange between donor and

recipient molecules in vitro (199). The lack of conservation of LEF-3 in baculoviruses might not be unexpected because, although homologs of alkaline nuclease are present in many organisms (see *Ac133*) and many have been reported to interact with an SSB (e.g., herpesvirus, ICP8 and lambda phage, red-beta), clear sequence relationships between the various SSBs are not evident (200). Insertion/deletion mutants of *lef-3* are lethal (62, 194).

***Ac68 (192aa:22.3kDa), (Bm56:134aa:15.8kDa).** Homologs of Ac68 are present in all baculoviruses. A frame shift in this gene did not affect transient late gene expression (135) and a deletion of Ac68 resulted in no major effects on AcMNPV production, although the lethal time was longer in larvae (201). In contrast, when the homolog in BmNPV (Bm56) was deleted in a bacmid, no effects on titers in cultured cells or in BV-injected larvae were detected, although the lethal time in larvae was longer. Although enveloped ODV were present, the polyhedra produced by the mutant bacmid were abnormal and lacked virions, suggesting that Bm56 is involved in polyhedron morphogenesis (202).

Ac69 (262aa:30.4kDa), (Bm57:262aa:30.4kDa) (MTase). Ac69 encodes a methyltransferase and orthologs are found in the genomes of all Group I NPVs, except OpMNPV and about one-half of Group II NPVs and one hymenopteran NPV. The homolog present in the hymenopteran NPV (NeseNPV) falls within an insect, rather than a baculovirus lineage. Homologs are found in a nudivirus (Hz-1) and a variety of insects, e.g., *Anopheles gambiae* (E = 8e-18) and other invertebrates and vertebrates. Ac69 was found to stimulate late gene transcription in a transient assay (135). The gene encodes a protein with RNA Cap (Nucleoside-2'-O)-Methyltransferase activity. AcMNPV, with a null mutation of the gene, replicated normally in cell culture (203). Similar results were observed for a knockout of the homolog (Bm57) in BmNPV (15).

Ac70 (290aa:34.4kDa), host cell-specific factor-1 (hcf-1). Homologs are present in only three other baculoviruses; two are close relatives of AcMNPV and their HCF-1 orfs are 99% (PlxyNPV) and 84% (RoMNPV) identical to that of AcMNPV, whereas the homolog in ClbiNPV is more distantly related (21% identical). HCF-1 was found to be required for transient expression of a late promoter-reporter gene by a late expression factor library in *T. ni* cells, but not SF-21 cells (204). AcMNPV with null mutations in hcf-1 were found to replicate normally in both Sf-21 cells and *S. frugiperda* larvae. However, in *T. ni* cells replication was impaired and in *T. ni* larvae the mutant showed a significantly reduced infectivity by intrahemocelic injection. Although oral infectivity was relatively normal in *T. ni* larvae, the insects died more slowly than when infected with wt (205). It was suggested that HCF-1 is a RING finger-containing protein that is dependent upon self-association and gene repression for its activity (206).

Ac71 (249aa:28.6kDa), (Bm58:249aa:28.7kDa), iap-2. Ac71 encodes an inhibitor of apoptosis-2 (*iap-2*) gene. Homologs of *iap-2* are found in the genomes of all Group I and most Group II NPVs, and as with all *iap* genes more distant relatives are found in many organisms. It is BV associated in AcMNPV (26). Deletion of *iap-2* had no effect on viral replication in cell culture; however, this may have been due to the presence of another apoptotic suppressor, *p35* (207). In contrast, deletion of *iap-2* (bm58) from the BmNPV genome indicated that it was required for replication in BmN cells (101). Evidence suggested that transfection of AcMNPV iap-2 into *T. ni* cells suppressed apoptosis by HearNPV infections, and although a recombinant HearNPV expressing iap-2 also suppressed apoptosis, BV production was not rescued (92). In *Epiphyas postvittana* NPV, the *iap-2* homolog was found to have anti apoptotic activity when expressed from a CMV promoter in *S. frugiperda* cells (93).

Ac72 (60aa:7.1kDa). Homologs of Ac72 are found in the genomes of all sequenced Group I NPVs, but not in other viruses. In BmNPV, this orf is 60 amino acids and was not characterized.

Ac73 (99aa:11.5kDa), (Bm59:99aa:11.5kDa). Homologs of Ac73 are found in the genomes of all sequenced Group I NPVs, but not in other viruses. It is BV associated in AcMNPV (26). It may be essential because insertion/deletion mutants of this gene in BmNPV (Bm59) could not be isolated (15).

Ac74 (265aa:30.6kDa), (Bm60:268aa:31.0kDa). Homologs of Ac74 are found in the genomes of all Group I and about half Group II NPVs, but is not present in hymenopteran or dipteran NPVs or GVs. It is BV associated in AcMNPV (26). Bm60 was found to be expressed as a late gene and was localized to both the cytoplasm and nucleus of infected cells (208). It was found to be associated with AcMNPV ODV (36), but not in HearNPV ODV (186). Deletion of Bm60 from BmNPV resulted in a reduction and delay in DNA synthesis, a reduction in BV production by about 10-fold, and a lengthening of the time to kill larvae (209).

Ac75 (133aa:15.5kDa), (Bm61:133aa:15.5kDa). Homologs of Ac75 are present in all lepidopteran NPV, GV and hymenopteran NPV genomes, but not in the dipteran virus genome. It was found to be associated with both BV and ODV of BmNPV and localized to the ring zone of infected cells (210). It may be an essential gene because insertion/deletion mutants of this gene in BmNPV (Bm61) could not be isolated (15).

Ac76 (84aa:9.4kDa), (Bm62:85aa:9.6kDa). Homologs of Ac76 are present in the genomes of all lepidopteran NPVs, GVs, and hymenopteran NPVs, but have not been reported in the dipteran virus genome. Ac76 localized to the ring zone late in infection. It is an essential gene, as deletion of ac76 resulted in a mutant bacmid able to produce DNA to normal levels, but was deficient in intra nuclear microvesicles and was unable to produce BV (211).

***Ac77 (379aa:44.4kDa), (Bm63:379aa:44.3.0kDa), Very late factor-1 (Vlf-1).** Homologs are found in all baculoviruses. The most closely related nonbaculovirus homologs are found in a number of eubacterial species, e.g., *Caldicellulosiruptor saccharolyticus* (E = 1e-06). Homologs are also reported in nudiviruses (170). VLF-1 is a member of the lambda integrase (212) family of proteins. Integrases are a large group of site-specific DNA recombinases that catalyze DNA rearrangements and are involved in the integration and excision of viral genomes and decatenation of newly replicated chromosomes. A feature of these enzymes is that a conserved tyrosine forms a covalent link with DNA during the cleavage process. VLF-1 was originally identified because it influences the hyperexpression of very late genes (213). It was found to bind near the regulatory region of very late genes (214). Whereas mutations to the region that affected very late gene transcription were not lethal, other mutations, including mutation of the conserved tyrosine, appeared to be lethal to the virus (215). VLF-1 appears to be a structural protein present in both BV and ODV (215) and localizes to the ends of nucleocapsids, suggesting that it is a structural protein (216) and is required for the production of nucleocapsids. Although *vlf-1* is an essential gene, an AcMNPV bacmid with *vlf-1* knocked out (216-218) was able to synthesize viral DNA at levels similar to control bacmids. However, the mutant produces tube-like capsids that appear to lack DNA. Characterization of a mutant of the conserved tyrosine indicated the nucleocapsids were unable to be released from the virogenic stroma, suggesting that the protein may be involved in a final step in the maturation of DNA (216). VLF-1 showed structure-dependent binding to DNA substrates with the highest binding affinity to cruciform DNA that mimics a structure common to recombination intermediates (219). See also Chapters 5 and 6.

Ac78 (109aa:12.5kDa), (Bm64:110aa:12.7kDa). This gene has homologs in all lepidopteran NPV and GV and also in two of the three hymenopteran genomes sequenced. It may be essential because interrupted mutants of this gene in BmNPV (Bm64) could not be isolated (15).

Ac79 (104aa:12.2kDa), (Bm65:104aa:12.2kDa). Homologs are present in all Group I, about half the Group II NPV, and three GV (Cp-, Ha- and XecnGV) genomes. It has homology to ascovirus orfs from *T. ni* (E = 1e-18) and *S. frugiperda* (2e-11), Chilo iridescent virus (E = 3e-10), and orfs from a variety of *Yersinia* sp (e.g., E = 2e-10), *Serratia* sp., and other bacteria. It is predicted to be homologous to an endonuclease in a number of these organisms. It has been suggested that Ac79 is a member of the UvrC superfamily of endonucleases that are involved in DNA repair (220). It may be essential because interrupted mutants of this gene in BmNPV (Bm65) could not be isolated (15). It was found to be associated with AcMNPV ODV (36).

***Ac80 (409aa:45.4kDa), (Bm66:403aa:44.9kDa), GP41, tegument protein.** GP41 is a tegument protein modified with O-linked N-acetylglucosamine, located between the virion envelope and capsid (221, 222). It was found to be associated with ODV by mass spectrometry (36, 186). Homologs are present in all baculovirus genomes. Based on the characterization of a ts mutant, Ac80 is an essential gene required for the egress of nucleocapsids from the nucleus (223). A deletion/insertion mutant of the homologous gene in BmNPV (Bm66) could not be isolated (15).

***Ac81 (233aa:26.9kDa), (Bm67:234aa:27.0kDa).** Homologs of this orf appear to be present in all baculovirus genomes. A homolog has also been reported in nudiviruses (170). The BmNPV homolog (Bm67) appears to be a late expressed nonstructural gene that localizes to the cytoplasm (224). When put through the HHpred program (59) that compares predicted proteins to known structures, Ac81 and its ortholog from NeseNPV showed relatedness to the HIV TAT protein with a probability of greater than 80%. It may be essential because an insertion/deletion mutant ortholog in BmNPV (Bm67) could not be isolated (15).

Ac82 (180aa:19.8kDa), (Bm68:181aa:20.1kDa), Telokin-like protein (TLP). *Telokin-like protein is not like telokin!* Homologs of Ac82 are found in the genomes of all lepidopteran NPVs and GVs. A protein called telokin is identical to 157 C-terminal amino acids of the myosin light chain kinase (MLCK), but is expressed independently. Telokin is the myosin binding fragment of myosin light chain kinase and is involved in muscle contraction. A polyclonal antibody prepared against smooth muscle telokin reacted with a protein from cell extracts of AcMNPV-infected Sf9 cells. This protein was called telokin-like protein. Clones that reacted with the antibody were isolated from a cDNA library of AcMNPV infected sf9 cells (225). The clones showed no sequence homology to telokin but when expressed in a pET vector, the product reacted with the telokin polyclonal antiserum. The AcMNPV sequence that produced the reactive protein contains portions of Ac82. The crystal structure of AcMNPV TLP was determined, but showed no similarity to telokin or any other characterized protein (226). Therefore, although this protein has been called telokin-like protein, its resemblance to telokin appears to be an artifact of the polyclonal antiserum. Consequently, information on a function for one of the few baculovirus specific proteins for which a crystal structure has been determined is still lacking. It is likely to be nonessential because when it was deleted in BmNPV (Bm68) the virus appeared to be normal, but production of BV and DNA replication was somewhat delayed. Although it showed nuclear localization and did not concentrate at the plasma cell membrane, it was found to be associated with the envelope/tegument of budded virions (227).

***Ac83 (847aa:96.2kDa), (Bm69:839aa:95.8kDa), VP91.** Ac83 encodes a virion capsid protein called vp91 that was originally characterized in OpMNPV (228). It has also been shown to be ODV associated in AcMNPV and CuniNPV by mass spectrometry (36, 86). Homologs are encoded by all baculovirus and are also found in nudiviruses (170) and possibly in several insect genomes, e.g., *Anopheles gambiae* (E = 5e-04). Ac83 is predicted to contain a chitin binding domain. It is likely an essential gene because insertion/deletion mutants of this gene in BmNPV (Bm69) could not be isolated (15).

Ac84 (188aa:21.7kDa). This orf is only found in a few other NPVs: PlxyNPV, ChchNPV, and RoMNPV, and TnSNPV. A homolog is also found in ascoviruses, e.g., *T. ni* ascovirus (E = 3e-10). It is not found in the BmNPV genome.

Ac85 (53aa:6.4kDa). This small orf encoding 53 aa is only found in two other NPVs that are AcMNPV variants: PlxyNPV and RoMNPV. This gene is not found in the BmNPV genome.

Ac86 (684aa:80.8kDa) (PNK/PNL). This gene encodes a protein with RNA ligase, polynucleotide 5'-kinase, and polynucleotide 3'-phosphatase activities and may be part of an RNA repair pathway (229). Homologs are only found in 5 baculovirus genomes, three are closely related to AcMNPV including AgMNPV, ApNPV, and RoMNPV, whereas the other is in a GV, SpliGV. A closely related orf is also found in a *T. ni* ascovirus (E = 2e-125). Ac86 appears to be a nonessential gene expressed early in infection (230). This gene is not found in the BmNPV genome.

Ac87 (126aa:15kDa), (Bm70:126aa:15.1kDa). This gene appears to be present in most Group I lepidopteran NPV genomes. It was suggested that the homolog in BmNPV (Bm70) might encode a capsid protein called p15 (231). It is likely nonessential, as an insertion mutant in BmNPV (Bm70) is viable (15).

Ac88 (264aa:30.1kDa), (Bm71:267aa:30.7kDa), CG30. Homologs of Ac88 appear to be present in the genomes of most Group I and II NPVs, and also may be present in a single GV (SpliGV). An orf in *Clostridium perfringens* showed significant similarity (E = 2e-06). Ac88 contains predicted zinc finger and leucine finger domains (232). It was found to be associated with AcMNPV (36), but not in HearNPV ODV (186). Deletion of this gene resulted in only subtle differences from wt (233).

***Ac89 (347aa:39kDa), (Bm72:350aa:39.3kDa), VP39.** This gene encodes the major capsid protein vp39. It is present in all baculovirus genomes. It was originally characterized in OpMNPV (234) and AcMNPV (235). It interacts with 38K (Ac98) (156). It is likely an essential gene because insertion/deletion mutants of this gene in BmNPV (Bm73) could not be isolated (15).

***Ac90 (464aa:53.9kDa), (Bm73:465aa:54.0kDa), LEF-4.** LEF-4 is an enzyme involved in RNA capping and is a component of the late baculovirus RNA polymerase (133). It is present in all baculovirus genomes and is also present in nudivirus genomes (170). This gene was originally identified as being essential for late transcription (123). LEF-4 was subsequently found to be an RNA capping enzyme (236, 237). The addition of an mRNA 5' cap structure involves the hydrolysis of the gamma phosphate of the 5'-triphosphate of the first nucleotide of pre-mRNA and the capping reaction that involves the transfer of GMP from GTP. The two reactions involve two different enzymatic activities: an RNA 5' triphosphatase to remove the terminal gamma phosphate and the addition of GMP by guanylyltransferase. These two activities are present on a single protein located at the N- and C-termini, respectively, in metazoans and plants. Although having similar activity, LEF-4 is unrelated to this category of capping enzyme, but is a member of metal dependent group of capping enzymes found in fungi and protozoa (236, 238, 239). The 5' cap structure appears to serve two roles. It protects the 5' end of the mRNA from degradation by exonucleases and it interacts with translation initiation factors, thereby facilitating the initiation of translation. Capping in eukaryotes involves an enzyme that associates with the highly repetitive carboxy terminal domain (CTD) of the β' subunit of RNA polymerase II. Because the baculovirus polymerase lacks a similar domain, it is likely that it evolved to include the enzyme as part of the RNA polymerase complex. However, assuming these reactions are free from exonuclease, it is not clear why LEF-4 is required for transcription in in vitro assays. These assays monitor RNA transcripts that would not need to be capped in order to be detected. This suggests that LEF-4 may play a structural role in the organization of the polymerase subunits, or it may have some other function. LEF-4 is an essential gene and could not be deleted (184). For more information see Chapter 6.

Ac91 (224aa:24.1kDa), (Bm74:154aa:17.3kDa). Homologs of this gene are found in genomes of all Group I lepidopteran NPV and two GV (CpGV and PlxyGV) genomes. It has an unusual predicted amino acid sequence: 31% proline and 18% ser/thr residues. In HearNPV the Ac91 homolog (ha80) was expressed first cytoplasmically and then in nuclei, but did not appear to be a structural protein of BV or ODV (240). Deletion of Bm74 causes few differences from wt and repair viruses in DNA synthesis or BV titers. However, the lethal time in larvae was longer by 14.7 hr (241).

***Ac92 (259aa:30.9kDa), (Bm75:259aa:30.9kDa), p33, sulfhydryloxidase (sox).** Ac92 is a flavin adenine dinucleotide (FAD)-linked sulfhydryl oxidase (242, 243). Orthologs of Ac92 are present in all sequenced baculovirus genomes, it is associated with BV and ODV, and it is an essential gene as viable recombinants deleted for this gene have not been isolated (243, 244). Ac92 is able to form a stable complex with the human tumor suppressor gene p53 when it was expressed in a baculovirus system. When expressed by itself, p33 shows diffuse cytoplasmic staining and punctate staining of nuclei. However, when co-expressed with p53, it exclusively localizes to nuclei. Expression of human p53 in Sf cells causes apoptosis that can be blocked by co-expression of baculovirus anti-apoptotic suppressors

p35 or OpIAP. However, co-expression of p53 with p33 elevated the induction of apoptosis about two fold. By proteomic analysis, p33 appears to be an ODV-associated protein in AcMNPV (36) and HearNPV (186). When put through the HHpred program (59) that compares predicted proteins to known structures, Ac92 and the ortholog from NeseNPV showed relatedness to sulfhydryl oxidase with a probability of almost 90%. Proteins with sulfhydryl oxidase activity have been implicated in the protection of cells from oxidative stress caused by apoptosis (245, 246).

Ac93 (161aa:18.4kDa), (Bm76:161aa:18.4kDa). This gene appears to be present in all lepidopteran and hymenopteran baculovirus genomes, but not in the dipteran (CuniNPV). Deletion is probably lethal as a virus with an insertion mutant of this gene in BmNPV (Bm76) could not be isolated (15).

Ac94 (228aa:25.5kDa), (Bm77:228aa:25.6kDa), ODV-E25 (p25, 25k). Homologs of ODV-E25 are present in all sequenced lepidopteran NPV and GV genomes, but not in those of hymenopteran or dipteran NPVs. The protein encoded by this gene was originally identified in OpMNPV, and immunogold staining with a specific antibody against Ac94 was localized to ODV envelopes (247). It has also been shown to be associated with ODV of AcMNPV and HearNPV by mass spectrometry (36, 186). It was also found associated with AcMNPV BV (26). The hydrophobic N-terminal 24 aa of AcMNPV ODV-E25 appears to be a nuclear targeting signal (140). Deletion is probably lethal as virus with an insertion/deletion mutant of this gene in BmNPV (Bm77) could not be isolated (15).

***Ac95 (1221aa:143kDa), (Bm78:1222aa:143.6kDa), DNA helicase (p143).** Homologs of DNA helicase are present in all baculovirus genomes. This gene was initially identified as a ts mutant that was unable to synthesize DNA at 33° (248). The defect was localized to a homolog of DNA helicase with a predicted mass of 143 kDa (249). P143 is required for transient DNA replication (38, 39) and shows ATPase activity and is able to unwind a DNA primer annealed to a larger DNA molecule in an ATPase-dependent manner (250). Helicase is dependent on an interaction with LEF-3 for transport to the nucleus (see Ac67, LEF-3) (191). Mutations in this gene have been implicated in affecting viral host range (251, 252). Deletion is probably lethal as deletion/insertion mutants of this gene in BmNPV (Bm84) could not be isolated (62).

***Ac96 (173aa:19.8kDa), (Bm79:182aa:21.0kDa), PIF-4.** This orf appears to be present in all baculoviruses and homologs also appear to be present in nudiviruses (170). The homolog in BmNPV (Bm79) is an ODV envelope associated protein (253) and was also found associated with the envelopes of BV (254). Deletion of Ac96 from a bacmid construct resulted in a virus that could replicate in cell lines, but not insects. Consequently it was concluded that Ac96 is a per os infectivity factor, the 4th such protein identified in the AcMNPV genome (254).

Ac97 (56aa:6.5kDa). This is a small orf (56 aa) and appears to be present only in AcMNPV. There is no homolog in BmNPV and it is positioned at the location of the apparent insertion of two bro (Ac2) homologs (see Ac2). The lack of this orf in closely related viruses may indicate that it is not a functional orf.

***Ac98 (320aa:38kDa), (Bm82:320aa:38.0kDa), 38K.** Ac98 encodes a predicted protein of 38k and orthologs are present in all baculovirus genomes. AcMNPV Ac98 interacts with itself, VP1054 (Ac54), VP39 (Ac89), and VP80 (Ac104) and is associated with BV and ODV nucleocapsids (156). In HearNPV it interacted with itself, ODV-E56,GP41, PIF-2 and PEF-3 (255). An AcMNPV bacmid deletion construct, although unable to produce infectious virions, was capable of DNA synthesis, but nucleocapsid formation was disrupted. Tube-like structures that appeared to lack DNA, but stained with an anti-vp39 antibody were observed (256). It has also been suggested that Ac98 is capable of stimulating transcription in a transient transcription assay (257). When put through the HHpred program (59) that compares predicted proteins to known structures (see Chapter 6), Ac98 showed relatedness to a set of enzymes including CTD phosphatases with a probability of almost 100%. Dephosphorylation of the CTD can negatively regulate RNA polymerase II by inhibiting RNA elongation (258, 259). Although it is required for nucleocapsid formation, it does not appear to be a structural component of ODV as determined by proteomic analysis (36, 186). However, it was detected in CuniNPV ODV (86).

***Ac99 (265aa:31kDa), (Bm83:265aa:31.1kDa) (LEF-5).** Homologs of lef-5 are found in all baculoviruses and are also present in nudiviruses (170). LEF-5 was originally identified as being required for transient late gene expression (260). It was demonstrated to interact with itself and to contain a domain similar to that of the RNA polymerase II elongation factor TFIIS (261). Subsequent investigations indicated that LEF-5 did not enable the baculovirus polymerase to transit pause sites, and it was concluded that it functions as an initiation factor, rather than an elongation factor (262). Deletion is probably lethal as interrupted mutants of this gene in BmNPV (Bm83) could not be isolated (62).

***Ac100 (55aa:6.9kDa), (Bm84:65aa: 8.1kDa), p6.9.** P6.9 is a small (55 aa) arginine/serine/threonine-rich DNA binding protein (263). Homologs appear to be found in all baculoviruses, but are apparently difficult to detect because of their small size and repetitive amino acid content (264). It was originally shown to be a DNA binding protein in a GV (265), and the homolog was isolated from AcMNPV (263). The high concentration of arginine and ser/thr residues is similar to protamines that are present in sperm nuclei of many higher eukaryotes and are involved in the production of highly condensed DNA. Protamines are also small molecules of 44-65 amino acids (266, 267). Arginine is positively charged, and the polyarginine tracts in protamines neutralize the phosphodiester backbone, whereas the ser and thr residues interact with other protamine molecules, thereby yielding a neutral, highly compact complex that is biochemically inert. P6.9 localizes to the nuclear matrix during infection (268). Using an AcMNPV bacmid deleted for p6.9, nucleocapsids were not produced although tube-like structures similar to those associated with the deletion of VLF-1 and Ac98 (see above) were observed. The mutant appeared to synthesize normal amounts of DNA, but did not produce infectious virus (269).

Ac101 (361aa:41.5kDa), (Bm85:362aa:41.6kDa), BV/ODV-C42. Ac101 encodes a capsid-associated protein of both BV and ODV (36). Homologs have been identified in all sequenced baculovirus genomes with the exception of the virus pathogenic for the dipteran, *Culex nigripalpus* (CuNiNPV) (45). It was reported to interact in a yeast two-hybrid assay and by native gel electrophoresis (270) with pp78/83 (Ac8) that has been shown to localize to the basal end region of nucleocapsids (50, 51). Evidence suggests that it binds to PP78/83 and transports it into nuclei (271) and is involved in actin polymerization (272). It also interacts with FP25 (Ac61) and Ac141 (273). Deletion of Ac101 from an AcMNPV bacmid appeared to affect nucleocapsid formation but, although lethal, did not appear to affect DNA synthesis (274). Deletion/mutation of Ac101 was also reported to be lethal by others (271, 275).

Ac102 (122aa:13.3kDa), (Bm86:123aa:13.5kDa), p12. Homologs of Ac102 are found in all lepidopteran NPV, and GV genomes, but not in hymenopteran or dipteran viruses. It appears to encode an ODV-associated protein (36) in AcMNPV, but not HearNPV (186). It is involved in the nuclear localization of G-actin (276). Deletion is probably lethal as insertion/deletion mutants of this gene could not be isolated in either AcMNPV (275) or BmNPV (Bm86) (15).

Ac103 (387aa:45.3kDa), (Bm87:387aa:45.4kDa), p45. Homologs of Ac103 are present in all lepidopteran NPV, GV, and hymenopteran NPV genomes. Deletion of Ac103 was lethal, no viable BV were detected, and the constructs appeared to be deficient in the envelopment of ODV and their incorporation into occlusion bodies (277).

Ac104 (691aa:79.9kDa), (Bm88:692aa:79.9kDa), vp80 capsid, vp87. Homologs of Ac104 are found in all Group I and II lepidopteran NPV genomes, but not in those of GVs or hymenopteran or dipteran NPVs. The homolog in OpMNPV encodes an 87 kDa capsid-associated protein of both BV and ODV (278). Evidence suggests that it is also capsid associated in AcMNPV (36, 279) and interacts with 38K (Ac98) (156). Deletion of Bm88 indicated that it is an essential gene required for normal nucleocapsid production (280).

Ac105 (553aa:65.6kDa), (Bm89:289aa:34.3kDa), He65. Homologs of Ac105 are found in the genomes of most Group I, about one-half the Group II and three GVs (Agse-, Ha-, and XecnGV). It is a member of a distinct family of ligases that includes editing ligases of trypanosomes, putative RNA ligases of many

species of archaea, and also baculoviruses and an entomopoxviruses (281). It is an early-transcribed gene (282). It may be involved in the nuclear localization of G-actin (276). It is probably nonessential as insertion/deletion of this gene in BmNPV (Bm89) had no apparent effect on infectivity (15).

Ac106/107 (243aa:28.3kDa), (Bm90:249aa:28.9kDa). These two orfs are likely a single gene as homologs are fused in many baculoviruses and were found to be joined when the region was resequenced in the C-6 strain (12). Homologs are found in all Group I and II lepidopteran NPV, GV and hymenopteran NPV genomes, but not in that of the dipteran NPV. Deletion is probably not lethal but insertion/deletion mutants of this gene in BmNPV (Bm90) appeared to affect the motility of infected larvae (60).

Ac108 (105aa:11.8kDa), (Bm91:105aa:11.8kDa). Homologs of Ac108 are present in all Group I and II NPV and all GV genomes except (PlxyGV). Although Ac108 was not found to be ODV-associated in AcMNPV (36) or HearNPV (186), the homolog in the *Antheraea pernyi* nucleopolyhedrovirus (p11) was found to be associated with ODV (283). It may be an essential gene, as virus with an insertion mutation in the BmNPV homolog (Bm91) could not be isolated (15).

***Ac109 (390aa:44.8kDa), (Bm92:391aa:45.0kDa).** Homologs of Ac109 are present in all baculovirus genomes. Evidence suggests that it is ODV-associated in AcMNPV (36) and *Helicoverpa armigera* NPV (Ha94-ODV-EC43) (186, 284) and also is BV-associated in AcMNPV (26, 285). Although DNA replication appeared to be unaffected, deletion of AcMNPV Ac109 was lethal and resulted in a block in nucleocapsid and polyhedron formation (286). Another study in AcMNPV indicated that polyhedra and virions were produced by Ac109 deletions, but the virus was not infectious (285).

Ac110 (56aa:6.8kDa). Homologs of Ac110 are present in all lepidopteran baculovirus genomes. It was not characterized in BmNPV.

Ac111 (67aa:8.2kDa), (Bm93:67aa: 8.2kDa). Homologs of Ac111 are present in genomes of all Group I, four Group II (Hear-, Heze-, Ld-, and LeseNPV) and two GVs (SpliGV and XecnGV). It may be an essential gene, as insertion/deletion mutants in the BmNPV homolog (Bm93) could not be isolated (15).

Ac112/113 (258aa:30.9kDa). These two orfs are likely a single gene as homologs are fused in many baculoviruses and were found to be joined when the region was resequenced in the C-6 strain (12). Homologs are present in three Group I variants of AcMNPV (Ac-, Plxy-, and RoMNPV), one Group II NPV (LdMNPV) and three GV (Spli- Hear-, and XecnGV) genomes. It shows highly significant homology to FPV217, a hypothetical protein of fowlpox virus (E = 8e-28). BLAST searches with FP217 only picks out baculovirus Ac112 homologs. A related orf is not present in BmNPV (16).

Ac114 (424aa:49.3kDa), (Bm94:424aa:49.4kDa). Homologs of Ac114 are found in all Group I NPV genomes. It shows some homology to a hypothetical orf of *Plasmodium falciparum* 3D7 (E = 5e-04). In AcMNPV it appears to be an ODV (36, 287) and BV associated protein (26). It is likely to be nonessential, as an insertion/deletion mutation of this gene in BmNPV (Bm94) had no apparent effect on infectivity (15).

***Ac115 (204aa:23kDa), (Bm95:204aa:23.0kDa), pif-3.** Homologs of *pif-3* appear to be present in all baculovirus genomes. It is also present in nudivirus genomes (170). Like other *pif* genes, *pif-3* is required for oral infectivity of insect but not for infection of cultured cells (81). It forms a complex with PIF-1 and PIF-2 (255). For more information see Ac138 (p74).

Ac116 (56aa:6.4kDa). Homologs of Ac116 are found only in four Group I NPVs (Ac-, Ro-, Bm-, and PlxyNPV). A related orf was not characterized in BmNPV because it was smaller (56aa) than the 60 aa cut-off of gene products that were characterized (16).

Ac117 (95aa:11kDa), (Bm96:95aa:10.9kDa). Homologs of Ac117 are found in the genomes of all Group I and seven group II NPVs. It is likely to be nonessential, as insertion/deletion mutants of this gene in BmNPV (Bm96) were similar to wt, although a slight effect on the motility of infected larvae was noted (60).

Ac118 (157aa:18.7kDa). Homologs are only found in three Group I NPVs (Ac-, Ro- and PlxyNPV) genomes. A related orf is not present in BmNPV.

***Ac119 (530aa:59.8kDa), (Bm97:527aa:59.8kDa), pif-1.** Homologs of *pif-1* are present in all baculovirus genomes and are also present in nudivirus genomes (170). This gene can be deleted and the mutant is still infectious for cultured cells, but is not orally infectious for insects (81). It forms a complex with PIF-2 and PIF-3 (255). For more information see Ac138 (p74).

Ac120 (82aa:9.5kDa), (Bm98:82aa:9.5kDa). Homologs of Ac120 are found in all Group I and most Group II genomes. It is likely to be nonessential, as an insertion/deletion mutation of this gene in BmNPV (Bm98) had no apparent effect on infectivity (15).

Ac121 (58aa:6.7kDa). Homologs of Ac121 are only found in the genomes of Bm- and PlxyNPV. It may be a transcriptional activator of some early genes, including IE1 and pp31 (288). However, it does not appear to activate late gene expression (135). It was not characterized by deletion analysis in BmNPV because at 57 aa it was below the cut-off for this study (16).

Ac122 (62aa:7.2kDa), (Bm99:61aa:7.1kDa). Homologs of Ac122 are present in all sequenced Group I genomes except EppoNPV. It is likely to be nonessential, as an insertion mutation of this gene in BmNPV (Bm99) had no apparent effect on infectivity (15).

Ac123 (215aa:25kDa), (Bm100:225aa:26.0kDa), Protein kinase 2 (PK2). Homologs of Ac123 are found only in BmNPV, PlxyNPV and RoMNPV and they are all over 98% identical to AcPK2. PK2 is closely related to translation initiation factor eIF 2α kinase (e.g., B. mori (E = 2e-20)), and the homology appears to be focused on the C-terminal region of the kinase domain (289). An AcMNPV mutant deleted for *pk2* displayed no differences from wt in its infectivity to cultured cells and insect larvae (289). In contrast, PK2 (Bm100) from BmNPV was found to be required for replication in BmN cells (101). In addition, novel eIF-2alpha kinase called BeK was identified from B. mori. It has a distinct N-terminal requlatory region not shared by other eIF-2alpha kinases. BmPK2 was found to be capable of inhibiting the enzymatic activity of BeK (290). AcMNPV PK2 was found to inhibit yeast and human eIF2α kinases (291). Insect cells infected with wt showed reduced eIF2α phosphorylation and increased translational activity that was not observed in cells infected with the *pk2* deletion mutant. It was suggested that this gene could be involved in a mechanism for inactivating a host stress response to virus infection (291). Sf9 cell infection by AcMNPV deleted for PK2 or by wt virus both encoding the p35 anti-apoptotic gene was found to induce the expression of BiP. BiP is a stress marker of the endoplasmic reticulum and a chaperone. It was observed, however, that the UV induction of eIF2alpha phosphorylation and the activation of caspase were mitigated more effectively by the wt virus than the mutant virus that lacks pK2, which is an eIF2alpha kinase inhibitor (292).

Ac124 (247aa:28.5kDa), (Bm101:244aa:28.1kDa). Homologs of Ac124 are present in the genomes of all sequenced Group I lepidopteran NPVs. It has been shown to be BV associated (26). It is likely an essential gene, as insertion/deletion mutants in the BmNPV homolog (Bm101) could not be isolated (15).

Ac125 (226aa:26.6kDa), (Bm102:227aa:26.6kDa), lef-7. Homologs of lef-7 are present in the genomes of all Group I, three Group II (Se-, Sf- and MacoNPV A) and two GVs (Ha- and XecnGV). Lef-7 is stimulatory for transient DNA replication (39, 293). When deleted, infection was unaffected in Tn368 cells, but in Sf21 and Se1c cells DNA replication was 10% of wt (294). Deletion of BmNPV lef-7 also caused a reduction in BmNPV DNA synthesis (62).

Ac126 (551aa:61.4kDa), (Bm103:552aa:61.8kDa), chitinase. Homologs of chitinase are present in genomes of all Group I (except AgMNPV), all Group II (except AdhoNPV) and four GVs (Agse, Cp-, Ha-, and XecnGV) and is phylogenetically clustered with a number of lepidopteran chitinases, i.e., it shows 63% aa sequence identity to *B. mori* chitinase. Comparison of BmNPV and *B. mori* chitinases indicated that, although closely related, they have different properties; the viral chitinase is retained in the cell and

functions under alkaline conditions, whereas the host enzyme is secreted and has reduced activity at higher pH (295). In conjunction with Ac127 (cathepsin), chitinase participates in the liquefaction of insects late in infection. It is a late expressed gene and its product is localized to the cytoplasm (296) and also is BV associated (26). When it is deleted along with Ac127 (cathepsin), insects remained intact for several days after death (297). Chitinase is localized to the endoplasmic reticulum in infected cells by KEDL, an endoplasmic reticulum retention motif (298, 299). The retention in the ER may prevent the premature death and liquefaction of infected insects, allowing the virus to continue to replicate. It is thought that the presence of chitinase and cathepsin assists in the dissemination of the virus by degrading the insect upon its death. The facility with which a virus (*Anticarsia gemmatalis* NPV) can be processed for use as a biocontrol agent has been attributed to its lack of these two genes, thereby allowing collection of the virus from intact rather than disintegrated insects (300).

Ac127 (323aa:36.9kDa), (Bm104:323aa:36.9kDa), cathepsin, vcath, a metalloprotease. Homologs of Ac127, cathepsin, have a similar distribution to Ac126 (chitinase) and are present in the genomes of all Group I (except AgMNPV), all Group II and four GVs (Agse-, Cp-, Crle-, and XecnGV). The baculovirus genes are closely related to insect cathepsins, i.e. Ac127 is 39% identical to an *Apis mellifera* cathepsin. The baculovirus cathepsin appears to participate along with chitinase in the liquefaction of infected insects (see Ac126) (301). When it is deleted along with Ac126 (chitinase), insects remained intact for several days after death (297). It has been suggested that Ac127 is synthesized in an inactive form that is activated upon death of the insect by lysosomal proteinases (302). It was subsequently demonstrated that AcMNPV and CfMNPV cathepsins are expressed are pre-proenzymes that are cleaved in infected cells (303).

Ac128 (530aa:60.6kDa), (Bm105:530aa:60.6kDa), gp64, gp67. *gp64* encodes a low pH activated envelope fusion protein, and homologs are present in all Group I genomes. It is one of the major distinguishing features of these viruses. It is thought that all Group I viruses use GP64 for the entry of BV into cells, whereas all other baculoviruses lack a *gp64* homolog and use the F protein (ac23 homolog) except for hymenopteran NPVs which lack both genes. In addition to the Group I NPVs, homologs of *gp64* are also found in thogotoviruses, which are members of the Orthomyxoviridae (304). GP64 (305-307) is a fatty acid acylated glycoprotein (308). Deletion of gp64 is lethal and results in viruses that replicate in a single cell, but cannot bud out and infect surrounding cells (309, 310). The postfusion structure of GP64 has been described (311).

Ac129 (198aa:22.1kDa), (Bm106:195aa:21.8kDa), **p24-capsid.** Homologs of Ac129 are present in the genomes of all Group I /II and GV genomes. Ac129 (p24) is associated with both BV and ODV of AcMNPV and OpMNPV (26, 312). Its presence in AcMNPV ODV was confirmed, however, the HearNPV homolog, He118, was not found associated with ODV (186). It is likely to be nonessential, as interruption of this gene with a transposable element in a strain of AcMNPV has been reported (313, 314). In addition, insertion/deletion mutations of this gene in BmNPV (Bm106), although viable, took slightly longer to kill insects than wt (60). Also, LdMNPV, the original strain sequenced, lacked this gene, whereas it is present in other strains (315).

Ac130 (106aa:12.1kDa), (Bm107:106aa:12.1kDa), gp16. Homologs of Ac130 are present in the genomes of all Group I, all Group II except (LdMNPV and LeseNPV), but are not present in those of GVs. In OpMNPV, the homolog (Op128) is glycosylated and localized near the nuclear membrane in the cytoplasm. Although it appeared to be associated with envelopes of nucleocapsids in the cytoplasm, it was not associated with either ODV or BV (316). It is likely to be nonessential, as an insertion mutation of this gene in BmNPV (Bm107) had no apparent effect on infectivity (15).

Ac131 (322aa:36.4kDa), (Bm108:315aa:35.4kDa), **calyx, polyhedron envelope (PE) protein, pp34.** Homologs of Ac131 are present in the genomes of all Group I and II NPVs. In addition, domains of PE may be present as fusion with segments of p10 in some GVs (see below). The calyx/polyhedron envelope

was originally found to be composed of carbohydrate (317); subsequently a phosphorylated protein component was identified (318) and was shown to be associated with the calyx/PE (319). Similar results were obtained for OpMNPV and it was also found to be associated with p10 fibrillar structures (320-323). In addition, in some viral genomes, genes are present that appear to be fusions of both PE and p10 protein domains (324, 325). The Ac131 encoded protein appears to be an integral component of the calyx/PE, and when the gene is deleted, polyhedra lack an intact calyx/PE, and have a rough surface showing cavities where virions have apparently been dislodged (326). It has also been reported to be BV associated (26) although what role it may play in this phenotype is not clear. The function of the calyx/PE appears to be to encase the occlusion body in order to enhance its stability. See Chapter 2 for additional information. The predicted size of Ac17 is longer than previously reported (12).

Ac132 (219aa:25.1kDa), (Bm109:220aa:25.2kDa). Homologs of Ac132 are present in all sequenced Group I genomes. Ac132 was identified as being associated with AcMNPV ODV(36) and BV (26). Bm109 was also reported to be ODV associated (327). It may be an essential gene, as a virus with an insertion mutation in the BmNPV homolog (Bm109) could not be isolated (15).

***Ac133 (419aa:48.3kDa), (Bm110:420aa:48.5kDa), Alkaline nuclease (AN).** Homologs of alkaline nuclease (AN) are found in all baculovirus genomes. They are also found in a variety of other viruses such as lambda phage and herpes viruses. In these viruses, the AN homolog associates with an SSB and has an exonuclease activity which generates 3' single-strand DNA ends that can participate in DNA recombination. In AcMNPV, AN interacts and co-purifies with the SSB LEF-3 and has both a 5'→3' exonuclease and an endonuclease activity (195, 196, 328). Deletion of Ac133 is lethal (329, 330). It is thought that AN is involved in DNA recombination. Homologs are also present in nudiviruses and hytrosaviruses.

Ac134 (803aa:94.5kDa) (p94). Homologs of Ac134 are present in the genomes of most Group I, three Group II (MacoA- MacoB-, Se- and SfMNPV), and three GVs (Agse-, Hear- and XecnGV). Homologs are found in several polydnaviruses, e.g., *Cotesia congregata* bracovirus (E = 7e-40) and entomopox viruses, e.g., *Melanoplus sanguinipes* entomopoxvirus (E = 1e-04), and several protozoa, some of which are insect-associated, e.g., the acetyl-CoA carboxylase 1 precursor of *Plasmodium yoelii* (E = 2e-05). The disruption of the p94 gene showed no effect on the ability of AcMNPV to infect *S. frugiperda* larvae by either the oral or intrahaemocelic route (331).

Ac135 (299aa:34.8kDa), (Bm112:299aa:34.5kDa), **p35.** P35 is an inhibitor of apoptosis, and homologs are limited to a few Group I NPVs closely related to AcMNPV. A homolog has also been reported in a GV of *Choristoneura occidentalis* (ChocGV) (324), and a variant (p49) is found in a Group II NPV (SpliNPV) genome (332, 333). Furthermore, a homolog most closely related to SpliNPV p49 has also been identified in an entomopox virus genome (334). P35 is able to block apoptosis in *S. frugiperda* cells caused by AcMNPV infection (335). Although deletion mutants are viable, they are severely compromised in BV production in Sf cells (335, 336). The crystal structure of p35 has been described (337, 338). For additional information, see Chapter 7.

Ac136 (240aa:27.3kDa), (Bm113:240aa:27.3kDa), p26. Homologs of p26 are present in the genomes of all Group I, all Group II except (SpliNPV), but are not present in those of GVs. Multiple copies of the gene may be present. Homologs are also found in the genomes of numerous pox viruses, e.g., Vaccinia (E = 0.15, 25% identity over 201 aa). Ac126 forms homodimers and is primarily a cytoplasmic protein (339). The examination of an AcMNPV deleted for p26 revealed no differences from wt in the cells and larvae tested (340). However, a deletion of p26 along with p10 and p74 resulted in polyhedra lacking virions (341).

Ac137 (94aa:10.3kDa), (Bm114:70aa:7.5kDa), p10. Homologs of p10 are found in the genomes of all Group I and II NPVs and most GVs, in some instances in multiple copies (342). They are also present in all hymenopteran NPV genomes. A p10 homolog has been characterized in an entomopox virus (343).

P10 was originally identified as a very late hyperexpressed gene (344) and therefore the p10 promoter has been used in expression vectors (345). P10 interacts with tubulin (346) and forms two different types of structures; microtubule-associated filaments, and tube-like structures that surround the nucleus (347). As noted above, p10 appears to be associated with the PE protein (Ac131) and in some viral genomes, genes are present that appear to be fusions of both PE and p10 protein domains (324, 325). Deletions of P10 result in polyhedra that resemble those produced by mutants lacking the calyx/polyhedron envelope protein (Ac131); they are fragile, have a rough surface showing cavities where virions have apparently been dislodged, and often show an incomplete calyx/polyhedron envelope (326, 348, 349). For more information, see Chapter 2.

***Ac138 (645aa:73.9kDa), (Bm115:645aa:74.0kDa), p74-pif.** P74 was the first member to be identified of proteins called per os infectivity factors that are required for oral infection of insects, but are dispensable for infection of cultured cells. Homologs of *p74* along with the other *pif* genes, *pif*-1 (*ac119*), -2 (*ac22*), and -3 (*ac115*) are present in all baculovirus genomes and are also found in genomes of nudiviruses (170). P74 was the first such protein identified and characterized (80, 350-353). Three other *pif* genes were identified in BmNPV (354) and subsequently their homologs were characterized in AcMNPV. AcMNPV deleted for *pif-1, -2,* or *-3* are not orally infectious for *T. ni* or *S. exigua* larvae based on feeding of 10,000 PIB of the deleted virus. They are also not orally infectious for *H. virescens*, except for the *pif-2* mutant that shows limited infectivity. In contrast, injection of 1 pfu, of the three deletion mutants, into third instar larvae of the three insect species caused over 80% mortality. In addition, PIF1, PIF2, and p74 mediate specific binding of occlusion derived virus to midgut cells, suggesting that they are directly involved in virus cell interaction as an initial step in infection (81). Although PIF-3 appears to be an ODV associated protein (355), it does not appear to be involved in specific binding and its function is not known. Both P74 (350) and a homolog of PIF-1 from *Spodoptera littoralis* NPV (356) also appear to be components of the ODV envelope. Co-infection with a wt- and a *p74*-deleted virus expressing *gfp* resulted in per os infection by the *gfp* expressing virus, suggesting the p74 did not have to be directly associated with a virus to facilitate per os infection. In addition, a 35-kDa binding partner for AcMNPV P74 was detected in extracts of brush border membrane vesicles from host larvae (*Spodoptera exigua*), but not from a nonhost (*Helicoverpa armigera* larvae) (353). The identity of this host protein has not been determined. By proteomic analysis, P74 was found associated with AcMNPV, HearNPV and CuniNPV ODV (36, 186, 86); however, the other PIF proteins showed differing associations,e.g., CuniNPV (PIF-1,2,3), AcMNPV (PIF-2) and HearNPV (PIF-1). Ac145 and Ac150 also may be *pif* genes (see below).

Ac 139 (449aa:52.6kDa), (Bm116:451aa:52.6kDa), ME53. Homologs of *ac139* are present in the genomes of all the lepidopteran NPVs and GVs, but have not been reported in hymenopteran or diperan baculovirus genomes. It is BV and ODV associated (357). One study indicated that AcMNPV deleted for this gene is not viable and fails to replicate its DNA and does not produce nucleocapsids. However, cells transfected with DNA from the mutant showed early stages of cpe, including nuclear enlargement and the formation of granular material in the nucleus (358). This suggests that the mutant is blocked in an early gene function. This is consistent with its original characterization as a major early gene (359). However, another study showed that deletion of Ac139 did not alter DNA replication, but results in a 1000-fold reduction in BV titer. In addition, it was found that it appears to be required both early and late in infection (357).

Ac140 (60aa:7.1kDa). This orf encodes 60 aa and is only found in AcMNPV.

Ac141 (261aa:30.1kDa), (Bm117:261aa:30.1kDa), exon0. Homologs of Ac141 are found in all lepidopteran NPVs, and orfs with low homology are also found in GV genomes. In AcMNPV it is associated with both BV and ODV nucleocapsids (360, 26) and interacts with BV/ODV-C42 (Ac101) and FP25 (Ac61) (273). Ac141 contains a predicted ring finger domain (361) that is a type of zinc finger comprising 40-60 residues that binds two zinc atoms and may be involved in protein-protein interactions. Deletion of Ac141 severely compromises BV production and results in virus that appear to be restricted to

cells initially infected, suggesting that Ac141 may be required for efficient egress of BV (360, 361). It appears to both co-localize with and co-purifiy with β-tubulin, and inhibitors of microtubules reduced BV production by over 85%. Therefore, it has been suggested that the interaction of Ac141 with microtubules might be involved in the egress of BV (362).

***Ac142 (477aa:55.4kDa), (Bm118:476aa:55.5kDa).** Homologs of Ac142 have been identified in all sequenced baculovirus genomes. Ac142 is associated with both BV and ODV virions, and deletion of Ac142 appeared to affect nucleocapsid formation but, although lethal, did not appear to affect DNA synthesis (274). Another study describing a different bacmid construct that deleted less of the Ac142 gene showed similar results except that some nucleocapsids appeared to be fully formed, but were un-enveloped in the nucleus and were not occluded (363). It is unclear whether the difference in the two studies was due to the removal of a 3' processing signal for the upstream Ac141 gene in the former investigation, or to the presence of a significant portion of the Ac142 orf in the latter study. A BmNPV deleted for Bm118 failed to produce BV and produced polyhedra lacking virions. There appeared to be a defect in nucleocapsid formation as elongated capsid-like particles apparently devoid of DNA were observed (364). Mass spectrometry also suggests that Ac142 is ODV-associated in three different viruses (36, 86, 186).

***Ac143 (90aa:9.7kDa), (Bm119:101aa:10.4kDa), odv-e18.** Homologs of Ac143 are present in the genomes of all baculoviruses. An antibody generated against an Ac143-GST fusion reacted with a protein of 18 kDa in the ODV envelope fraction, and Ac143 was named ODV-E18 (365). Ac143 and its HearNPV homolog were found in surveys of ODV-associated proteins by mass spectrometry (36, 186). No BV is produced when Ac143 is deleted (366). Ac143 was found to be BV associated in a proteomic analysis (26).The predicted size of Ac17 is longer than previously reported (12).

***Ac144 (290aa:33.5kDa), (Bm120:290aa:33.5kDa).** Homologs of Ac144 are present in all sequenced baculovirus genomes. Ac144 was named ODV-EC27; however, other data suggests that it is present in both BV and ODV and has a molecular weight of 33.5 kDa (see below). Mass spectrometry also suggests that Ac144 is ODV-associated in three different viruses (36, 86, 186). A variety of investigations have been conducted on Ac144. Initially, it was confirmed that its transcript initiates at a late promoter element (365). Later, it was suggested that it is a multifunctional cyclin and may be involved in regulating the cell cycle during virus infection (367). It was reported to interact in a yeast two-hybrid assay with Ac101 described above (also named C42) and with both Ac101 and p78/83 (Ac9) in native gel electrophoresis assays (270). Although this orf was designated as ODV-EC27, another investigation using an HA-tagged Ac144 recombinant virus and anti-HA monoclonal antibodies found that Ac144 was expressed as an ~ 33.5 kDa protein which conforms to the predicted MW (274). In addition, it was found to be BV associated (274, 26). It was also found that deletion of Ac144 appeared to affect nucleocapsid formation but, although lethal, did not appear to affect DNA synthesis (274).

Ac145 (77aa:8.9kDa), (Bm121:95aa:11.0kDa) and 150 (pif?). These two genes encode small proteins (~9 and 11 kDa, respectively) that are related to one another (23% aa sequence identity) and are also related to a gene encoding an 11-kDa protein in an entomopox virus of *Heliothis armigera*. Close relatives of Ac145 are found in all baculovirus genomes including lepidopteran NPVs and GVs, and hymenopteran NPVs, but not the dipteran NPV. In contrast to Ac145, close relatives of Ac150 are only found in a few NPVs closely related to AcMNPV. However, it shows significant homology (E = ~10^{-3}) to predicted proteins from several dipteran insects. Ac145 and 150 are predicted to encode a domain thought to bind to chitin (368). In one study (212), deletion of Ac145 led to a sixfold drop in infectivity in *T. ni,* but not *H. virescens* larvae. An effect of deletion of Ac150 was not detected. Deletion of both genes causes a major (39-fold) reduction of infectivity for *H. virescens*. Injection of BV of the double mutant intrahemocoelically was as infectious as wt suggesting that these genes play a role in oral infection. These properties suggest that Ac145 and Ac150 are pif genes. Products of *ac145* and *ac150* were found to be associated with both BV and ODV. In another study (369), occluded virions deleted for Ac150 were found to be significantly less

virulent when administered per os than the wt virus in *Heliothis virescens*, *S. exigua* and *T. ni* larvae. Evidence suggested that the mutant had a reduction in its ability to establish primary infections in midgut cells. The Ac145 homolog in HearSNPV was found to bind to chitin (370). For related genes see Ac138 (p74).

Ac146 (201aa:22.9kDa), (Bm122:201aa:22.9kDa). Homologs of Ac146 are present in the genomes of all lepidopteran NPV and GV genomes, but are not present in those of hymenopteran or dipteran. When Bm122 was fused with gfp, nuclear localization was observed (371). Ac146 is likely an essential gene, as infectious BV was not produced by an mutant deleted for the gene (372).

Ac147 (582aa:66.9kDa), (Bm123:584aa:66.9kDa), Immediate early gene-1 (IE-1). Homologs of IE-1 have been identified in all Group I and II genomes sequenced. They also appear to be present in all GV genomes, but the homology is very low, e.g., XcGVorf9 vs. Ac147 show about 10% amino acid sequence identity. However, the orientation and position of XcGV orf9 relative to more conserved orfs is similar to Ac147, suggesting that the limited homology might be real. In addition, the limited identity is located in conserved regions that are identified by other more convincing alignments, e.g., Ac-Ie1 vs. Ld-Ie1 (23% identity). Part of the IE-1 population is called IE-0 and is translated from a larger spliced mRNA, which is the only major spliced transcript described for baculoviruses (see below Ac147-0). IE-1 was originally identified because of its ability to transactivate early promoters of AcMNPV (118). The ability of IE-1 to transactivate transcription is greatly enhanced when the activated gene is linked to *hr* sequences (373). It also may participate in the negative regulation of some genes (374). IE-1 is required for transient DNA replication (38, 39). Whereas deletions of either IE-1 or IE-0 can support infectious virus production, inactivation/deletion of both these genes is lethal (375). Similar results were reported for BmNPV (62).

Ac147-0 (636aa:72kDa) (ie-0). Part of the IE-1 population is called IE-0 and is translated from a larger spliced mRNA, which is the only major spliced transcript described for baculoviruses and in AcMNPV results in an additional 54 amino acids at the N-terminus of IE-1 for a total of 636 amino acids (376). AcMNPV IE-1 is present as a homodimer but also can form a heterodimer with IE-0, and either IE-1 or IE-0 can support infectious virus production; however, there were subtle differences in timing of events and production of BV and polyhedra, depending on which gene is being expressed, suggesting that both *ie-0* and *ie-1* are required for wt levels of infection. As described above, *ie-0* can be eliminated, as long as IE-1 is being produced (375). In contrast, in LdMNPV only the spliced form is able to transactivate transient transcription and DNA replication (377).

***Ac148 (376aa:40.9kDa), (Bm124:375aa:41.3kDa), odv-e56, PIF-5.** Homologs of odv-e56 are present in the genomes of all baculovirus and are also present in nudivirus genomes (170). ODV-E56 localizes to the envelopes of occluded virions (378) in AcMNPV and other baculoviruses (379) and has also been reported to be associated with AcMNPV BV (26). An insertion mutant, in which the lacZ gene was placed in frame at about amino acid 139 (out of 376) was viable (378). A lacZ gene insertion near the N-terminus of the BmNPV homolog (Bm124) likely inactivated the gene and appeared to severely compromise the virus (15). Ac148 and its homologs in HearNPV and CuniNPV were found to be ODV associated (36, 86, 186). Deletion of Ac148 from a bacmid construct resulted in a virus that could replicate in cell lines, but not insects. Consequently it was concluded that Ac148 is a per os infectivity factor, the 5th such protein identified in the AcMNPV genome (380, 381). Similar conclusions were drawn for a BmNPV deletion of Bm124 (382).

Ac149 (107aa:12.4kDa), (Bm125:106aa:12.3kDa). Homologs of Ac149 are present in 4 other Group I viruses closely related to AcMNPV (Bm-, Mavi-, Plxy-, and RoNPV). It is likely to be nonessential as BmNPV with insertion/deletion mutations of this gene (Bm125) were infectious (15).

Ac150 (99aa:11.2kDa), (Bm126:115aa:13.4kDa), pif?. Ac150 is related to Ac145. In contrast to Ac145 homologs that are found in lepidopteran NPV and GV and hymenopteran NPV genomes, Ac150 is only found in a few group I NPVs closely related to AcMNPV. in AcMNPV, deletion results in less infectivity by

occluded virions for larvae (369). In BmNPV, deletion resulted in no apparent difference in BV production of mean lethal dose by occlusion bodies although the lethal time was extended somewhat (383). For more information, see Ac145 and Ac138 (p74).

Ac151 (408aa:47kDa), (Bm127:422aa:48.8kDa), ie-2/ie-n. Homologs of *ie-2* are limited to the genomes of all Group I lepidopteran NPVs. IE-2 contains a predicted RING finger domain and shows significant levels of similarity to a protein of *Trichomonas vaginalis* (E = 1e-06), an anaerobic, parasitic flagellated protozoan. IE-2 was found to augment activation by IE-1 (384-386). BmNPV IE-2 interacts with itself (387). IE-2 was required for optimal origin specific plasmid DNA replication in Sf-21 cells, but had little effect in Tn-368 cells (204). *Ie-2* deletion mutants behaved differently in Sf-21 cells in which the infection was delayed vs. Tn-5B1-4 cells, in which the infection was not delayed. In insect larvae, the mutant viruses were significantly less infectious than wt, which appeared to be due to a lack of virions in the occlusion bodies (388). IE-2 may also be involved in cell cycle regulation (389).

Ac152 (92aa:10.8kDa). Homologs of Ac152 are present in the genomes of four Group I NPVs closely related to AcMNPV, and three Group II NPVs. It is associated with the nuclear localization of G-actin (276). A homolog is not found in the BmNPV genome.

Ac153 (321aa:37.4kDa), (Bm128:309aa:36.1kDa), pe38. *Ac153* homologs have an unusual distribution being found in all Group I NPV and four GV genomes. Duplicate copies appear to be present in some of the genomes. Ac153 was originally identified because of its early transcription profile and the presence of predicted zinc finger and leucine zipper motifs (390). However,in OpMNPV it was shown to be expressed as full length (34 kda) and truncated (20 kda) forms with the larger variant functioning as a transcriptional transactivator of an early promoter (391). In addition, it appears to activate DNA replication in transient assays (38). Deletion of *pe38* results in a reduction in the expression of several genes, a delay in DNA replication, a 99% reduction in BV production, and reduced levels of DNA synthesis and was less orally infectious in larvae (392, 393).

Ac154 (81aa:9.4kDa), (Bm129:77aa:8.9kDa). Homologs of Ac154 are present in 4 other Group I viruses closely related to AcMNPV (Bm-, Mavi-, Plxy-, and RoNPV). It is likely to be nonessential, as an insertion mutation of this gene in BmNPV (Bm129) had no apparent effect on infectivity (15).

Hrs (homologous regions). In AcMNPV, *hrs* are comprised of repeated units of about 70-bp with an imperfect 30-bp palindrome near their center. They are repeated at eight locations in the genome with 2 to 8 repeats at each site. They are highly variable, and although they are closely related within a genome, they may show very limited homology between different viruses. For example, in the CpGV genome, tandem repeated sequences are not evident, although a 75-bp imperfect palindrome is present at 13 different locations on the genome (394). In addition, in the TnSNPV (Group II) sequence, *hrs* were not found (395). *Hrs* have been implicated both as transcriptional enhancers and origins of DNA replication for several baculoviruses (373, 396-400). They bind the transcriptional activator IE-1 (Ac147) (401-403) and this binding may cause IE-1 to localize into foci which may be a prelude to replication loci (404). *Hrs* contain a high concentration of camp and TPA response elements (CRE and TRE) that bind cellular transcription factors and stimulate RNA polymerase II dependent transcription and enhance activation by IE-1 (405). It has been shown that deletion of individual *hrs* or combinations of two *hrs* does not appear to affect virus replication in cultured cells (406).

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Chapter 13

Selected baculovirus genes without orthologs in the AcMNPV genome: Conservation and function

Below is a non-inclusive list of baculovirus genes that are not present in the AcMNPV genome, but that either have homology with well-characterized genes from other organisms, or that have been investigated in baculoviruses. Following this list is a summary of investigations on each gene.

- DNA ligase
- dUTPase
- Enhancin
- Helicase-2
- Iap-3
- PARG
- PARP
- Photolyase
- Ribonucleotide reductase Large subunit
- Ribonucleotide reductase Small Subunit
- Trypsin-like
- V-TREX

DNA Ligase. A DNA ligase would be involved in the ligation of Okazaki fragments during lagging strand synthesis. Homologs of DNA ligase are present in all sequenced granulovirus genomes and two NPV genomes (LdMNPV and MacoNPV-B). The DNA ligase of LdMNPV was characterized and found to be capable of ligating double-stranded synthetic DNA substrates containing a single nick [1]. A striking feature of the baculovirus ligase homologs is that they are always (except MacoNPV-B) accompanied with a helicase homolog that is not found in any of the genomes lacking ligase. This helicase is related to the PIF1 family [1] (note: this is not a per os infectivity factor). Members of this family have a preference for RNA-DNA hybrids and could be involved in the maturation of Okazaki fragments [2]. This may involve displacement of the RNA primer producing an RNA flap that would then be cleaved by a flap endonuclease (FEN) [3] or digested by a 5' to 3' exonuclease. DNA polymerase would then fill in the gap by extending the Okazaki fragment and the ligase could join the fragments.

dUTPase. Deoxyuridine triphosphate (dUTP) can be mutagenic if incorporated into DNA. The enzyme dUTPase dephosphorylates dUTP to dUMP, which is a substrate for thymidine biosynthesis. Homologs of dUTPase are present in 10 NPV (all Group II except for OpMNPV) and two GV genomes [4]. Baculoviruses may have incorporated this gene to either supplement or substitute for the host gene. The viruses that encode a *dutpase* homolog also normally encode both subunits of ribonucleotide reductase (RR) (see below). The presence of RR may have selected for the incorporation of *dutpase* in order to mitigate the production of the dUTP mutagen by ribonucleotide reductase.

Enhancin. Metalloproteinases are endopeptidases that contain divalent cations as integral components of their structure [5]. Enhancins are members of this proteinase group and are encoded by a few lepidopteran NPVs (e.g., Ld-, Cf-, and MacoNPV) and GVs (e.g., Ag-, As-, Tn-, XcGV). In one study of TnGV, enhancin was estimated to comprise up to 5% of the mass of occlusion bodies [6]. In LdMNPV, enhancin was found to be associated with ODV [7]. Enhancin genes are often present in multiple copies, e.g., the XecnGV genome has four copies [8]. In LdMNPV, which encodes two enhancins, deletion of either results in a 2- to 3-fold reduction in potency, whereas deletion of both caused a 12-fold reduction [9]. Enhancin is thought to facilitate baculovirus infection by digesting the peritrophic membrane (PM). The PM forms a barrier in insect guts that prevents the ready access of pathogens to the epithelial cells. The PM is rich in chitin, and insect intestinal mucin and enhancins appear to target the degradation of intestinal mucin, thereby facilitating access of virions to the underlying cells [10].

Enhancins shows sequence homology with high levels of significance (e.g., E = 3e-29) to predicted proteins of some pathogenic bacteria, e.g., *Clostridium botulinum*, and a variety of *Bacillus* (e.g., *B. anthracis*) and *Yersinia* (e.g., *Y. pestis*) species. To investigate their function, enhancins from *B. cereus*, *Y. pseudotuberculosis*, or TnGV were cloned into a construct of AcMNPV that yielded occluded viruses. Although the LD50 of these constructs was found to be about half of wt, only the construct expressing the TnGV enhancin caused a reduction in survival time. In addition, the bacterial enhancins failed to degrade insect intestinal mucin. It was suggested that the bacterial enhancins may have evolved an activity distinct from their viral homologs [11].

Helicase-2. A second helicase homolog has been found in 10 GV and two NPV genomes [4]. The homology to one of the NPVs, from *Spodoptera littoralis* (SpliNPV)-ORF 40, is minimal. The *hel-2* gene from LdMNPV [12] is related to a yeast helicase that is important in recombination and repair of mitochondrial DNA. It had no effect on DNA replication in a transient replication assay and could not substitute for helicase-p143 [1]. With one exception, *Mamestra configurata* NPV (MacoNPV-B) (and the limited homology of SpliNPV-ORF40 described above), the *hel-2* and *DNA ligase* genes (see above) are found in the same genomes (predominantly GVs), suggesting that they may participate in the same metabolic pathway in these viruses (see Chapter 5).

Iap-3. Inhibitor of apoptosis-3. Although 5 lineages of *iap* genes have been identified in baculoviruses, the *iap-3* lineage is the most well characterized and is a powerful inhibitor of apoptosis in certain cell lines. It is not found in the AcMNPV genome, although related *iap* genes are present. This lack of *iap-3* is likely compensated by the presence of *p35*, another apoptotic inhibitor. Members of the *iap*-3 lineage are found in Group I, II, GVs and hymenopteran NPVs. *Iap-3* genes are closely related to *iap* genes of insects. OpMNPV IAP-3 is 57% identical to IAP from *B. mori* indicating that the *iap* gene was likely captured by viruses on one or more occasions. In addition, *iap* from *S. frugiperda* has similar properties to IAP-3 in terms of its structure and function [13]. For additional information, see Chapter 7.

Metalloproteinase. As described above, metalloproteinases are endopeptidases that contain divalent cations. Baculoviruses encode three distinct metalloproteinases, cathepsin, enhancin, and a stromelysin1-like metalloproteinase. Although cathepsin homologs are found most lepidopteran group I and II NPVs, they are only found in four GV genomes and are not present in the hymenopteran and dipteran viruses. However, there are other enzymes encoded in GVs that might compensate for the lack of cathepsin. One such enzyme is a metalloproteinase that has homologs in all sequenced GV genomes, but is not present in NPV genomes. They have about 30% amino acid sequence identity to a catalytic domain in a stromelysin1 metalloproteinase of humans and sea urchins. The GV enzyme lacks a signal peptide and a cysteine switch that maintains the other enzymes in an inactive form. The stromelysin1-like metalloproteinase from XcGV was characterized and found be capable of digesting proteins and was inhibited by metalloproteinase inhibitors [14]. It is possible that the universal presence of metalloproteinase homologs in the GV genomes is involved in assisting in their viral transmission by facilitating the disintegration of cells after the GV replicative cycle is complete.

PARG. All sequenced Group II genomes encode homologs of Poly (ADP-ribose) glycohydrolase (PARG) [15, 16]. PARG is the primary enzyme responsible for the catabolism of poly(ADP-ribose) *in vivo* (see PARP below). It catalyzes the hydrolysis of glycosidic (1'–2') linkages in poly(ADP-ribose) to produce ADP-ribose [17]. Therefore, whereas PARP stimulates a variety of processes (see below), PARG reverses the products of PARP. In HaSNPV, PARG (Ha100) was ODV associated [16].

PARP. A homolog of poly (ADP-ribose) polymerases (PARP) has only been reported in a single baculovirus genome, *Anticarsia gemmatalis* (AgMNPV), Ag31 [18] [19]. PARP is an enzyme found in nuclei that is activated by DNA strand breaks and uses NAD+ as a substrate to synthesize polymers of ADP-ribose on acceptor proteins that are involved in the repair of single strand breaks in DNA by activating and recruiting DNA repair enzymes. It is also involved in telomere elongation, chromatin structure, and the transcription of a variety of genes involved in immunity, stress resistance, hormone responses, and the possible silencing of retroelements [20, 21]. It may also be involved in the regulation of a mitochondrial protein that induces apoptosis [22]. PARP is a caspase-3 substrate and its cleavage is used as a measure of apoptosis.

Photolyase. Homologs of photolyase genes have been found in the genomes of Group II baculovirus that are members of a lineage that infects insects of the subfamily Plusiinae of the family Noctuidae [23-25]. These enzymes are involved in the repair of DNA damage caused by ultraviolet light. *Chrysodeixis chalcites* encodes two photolyase genes that are predicted to encode proteins with 45% amino acid sequence identity. When both were tested, only one copy showed photoreactivating activity [26]. Transfection of egfp fusions of photolyase genes into *T. ni* cells, resulted in fluorescence localized to chromosomes and spindles and other structures associated with mitosis. Baculovirus infection of the transfected cells caused fluorescence to localize to the virogenic stroma [27]. The incorporation of an algal virus photolyase gene as a means to cause resistance to UV inactivation of AcMNPV has been described. However, although BV survival was increased after exposure to UV light, occluded virion survival was not affected [28].

Ribonucleotide reductase. Ribonucleotide reductase is a heterodimer composed of large and small subunits (RR1 and RR2, respectively). It is involved in the catalysis of ribonucleotides to deoxyribonucleotides as a pathway for providing nucleotides for DNA synthesis. Well documented RR1 and RR2 genes have been reported in the genomes of three GVs, 10 distinct Group II NPVs, and a single Group I NPV (OpMNPV) [4]. Two different RR2 genes have been reported for LdMNPV [12]. Based on the phylogeny of baculovirus RR1 genes, it has been postulated that two different capture events resulted in baculoviruses obtaining this gene [29]. One source was from a bacterium for the OpMNPV and LdMNPV RR1 gene lineage, whereas the other lineage (e.g., *Spodoptera exigua* MNPV (SeMNPV)) appears to have been derived from eukaryotes, most likely insects. The two RR2 genes from LdMNPV appear to be derived independently, one from each different source, rather than via gene duplication. No enzymology has been described for baculovirus RR and it is not known whether they have enzymatic activity or how they integrate with or substitute for the homologous host enzymes.

Trypsin-like. Although hymenopteran lack homologs of chitinase and cathepsin, they all encode a trypsin-like protein (e.g., Nese7) [30] that shows high levels of aa sequence identity (e.g., 50%) to insect trypsin-like homologs. It is possible that the presence of this enzyme compensates for the absence of chitinase and cathepsin and facilitates the release of virus from infected gut cells into the environment and to provide inoculum for the re-infection of other gut cells.

V-TREX (Viral three-prime repair exonuclease). A gene with homology to 3' to 5' exonucleases from other systems has been identified in three Group I NPVs, AgMNPV, CfMNPV and AnpeNPV. The enzyme from both AgMNPV and CfMNPV demonstrated to 3' to 5' exonucleolytic activity. It is thought that they may be involved in DNA repair [31, 32].

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Glossary

Bacmid. Bacmids are baculovirus genomes that contain a bacterial origin of replication so that they can replicate in bacteria as a plasmid [1]. The term is derived from a contraction of baculovirus and plasmid (Luckow, pers. comm.). The original construct contained the AcMNPV genome, a bacterial origin of replication, the target site for the bacterial transposon Tn7, and a selectable kanamycin resistance marker gene, and the lacZ gene all in the polyhedrin locus. This construct allowed for the manipulation and recombination via transposition of the viral genome in bacteria; however, the construct is viable upon transfection into insect cells.

Baculoviridae. A family of occluded viruses pathogenic for insects and possibly other invertebrates. They have large circular, supercoiled, double-stranded DNA genomes and replicate in nuclei.

Basal lamina. This is a fibrous structure that separates the insect midgut epithelium from the hemocele. It is a barrier that baculoviruses might have to cross to cause a systemic infection.

BmN, (BmN 1, 4, 5). Variants of a cell line derived from *Bombyx mori* that are permissive for infection of BmNPV. The original cell line was isolated by Dr. T. D. Grace. The derivation of the different subclonal lines is ambiguous and is discussed in [2].

Burst Sequence. This is an A/T-rich regulatory sequence between the promoter and translational start site of the very late hyperexpressed genes, polyhedrin and p10. Evidence suggests that it binds VLF-1 [3].

Budded virus (BV). A type of baculovirus that buds out of infected cells and spreads the infection within an insect and within cell culture. The BVs derive their envelope from modified cell membranes.

Caspase. A category of proteases activated in the apoptotic pathway. There are two types: initiator and effector. Initiator caspases are regulatory and cleave and thereby activate effector caspases. Effector caspases carry out the apoptotic program.

E (expect) value. The E-value is an indication of the statistical significance of a specific pairwise sequence alignment and reflects a combination of the number of sequences in a database and the scoring system employed. The lower the E-value, the more significant the relatedness. An alignment with an E-value of 0.001 indicates that this amount of relatedness has a 1 in 1000 probability of occurring by chance alone. Although E-values often indicate convincing relatedness, they can be distorted by repeated amino acids and a variety of other factors.

Encapsulation. Encapsulation is a defense response in insects that is directed against objects that are too large to be phagocytose. It involves the accumulation of hemocytes that form a capsule around the object and often is accompanied with melanin deposition.

Enhancin. See Metalloproteinases

Errantivirus. Errantiviruses are insect retroelements that encode an env gene and their name is derived from Latin *errans*, to wander. Although similar to retroviruses, they have not been included within the Retroviridae because they are a distinct lineage, and evidence that they are infectious is indirect (see Chapter 11). Their env gene is related to the baculovirus F protein [4] [5].

F protein. This protein is thought to be the fusion protein of most baculoviruses with the exception of Group I, which use gp64 and hymenopteran viruses that do not encode homologs of either gp64 or F. F protein homologs are present, as env proteins in insect retroviruses, also called errantiviruses. Homologs are also present in some insect lineages.

Few Polyhedra (FP) mutants. Few polyhedra (FP) mutants are a readily observable baculovirus phenotype that result in reduced numbers of polyhedra and an elevated titer of budded virus. Such mutants often contain an insert in the *fp* gene (ac61), although the phenotype can result from mutations elsewhere in the genome.

GP64. The envelope fusion protein used by Group I NPVs. It is related to the envelope fusion protein of a group of arthropod borne orthomyxoviruses, the thogotoviruses.

Granulosis viruses; granuloviruses (GV). A lineage of baculoviruses pathogenic for Lepidoptera, which normally have a single virion per ovoid-shaped occlusion body.

Group I. One of two major lineages of lepidopteran NPVs; it is distinguished from other baculoviruses by using a different envelope fusion protein, gp64. Several other genes are also unique to this lineage.

Group II. One of two major lineages of lepidopteran NPVs; members are thought to use a fusion protein (F) to initiate infection

Homolog (homologous protein; ortholog, paralog). Homolog is an inclusive term that indicates that two proteins are evolutionarily related. Homologs are divided into orthologs and paralogs. **Orthologs** (ortho = exact) have homology resulting from speciation and are directly related evolutionarily to one another from a common ancestor via vertical descent, whereas **paralogs** (para = parallel) show relatedness that results from gene duplication. Often, this leads to one copy evolving a different function. Due to the presence of more than one lineage and the possible loss of one duplicate in some lineages, the phylogeny of paralogous proteins may not reflect a direct phylogenetic relationship via a common ancestor.

Hrs, homologous repeated sequences. These are often located at several sites in a baculovirus genome, which have been implicated as origins of DNA replication and transcriptional enhancers.

Hytrosavirus. Hytrosaviruses are also known as the salivary gland hypertrophy viruses and have been characterized from several of Diptera including the tsetse fly, the vector for sleeping sickness. They are non-occluded and contain large, circular, double stranded DNA genomes within enveloped rod-shaped nucleocapsids. They appear to infect the salivary gland and although not particularly virulent, they can result in a significant reduction in reproductive fitness. They encode several per os infectivity factor] proteins related to those of baculoviruses and nudiviruses. The name is derived from the symptoms (hypertrophy of the salivary gland) [7].

Inhibitor of apoptosis (IAP). The IAP gene family was originally discovered in baculoviruses [8]. Subsequently homologs have been found in almost all baculovirus and also entomopox and irridoviruses, and they are also widely distributed in eukaryotes, from yeast to mammals. IAP sequences have a number of distinguishing domains. These include baculovirus IAP repeat (BIR) domains of about 70 amino acids. BIR domains are often present in multiple copies with two copies present in many baculovirus IAPs and up to three copies in some cellular IAPs. A zinc (RING) finger domain is also often present near the C-terminus of the protein. For more information see Chapter 7.

LEF, late expression factor. In AcMNPV, these are factors that are involved in transient DNA replication or late transcription.

Melanization. In insects, melanization involves the synthesis and deposition of melanin at the site of injury. It is regulated by a cascade of serine proteases that cleave and activate prophenoloxidase (to phenoloxidase) that is then able to catalyze the oxidation of phenols (e.g., tyrosine) to quinones (nonaromatic ring compounds), which then polymerize and form melanin [9].

Metalloproteinases. Metalloproteinases are peptidases that contain divalent cations as integral components of their structure [10]. Baculoviruses encode a several members of this group of enzymes. They include enhancin, which is thought to enhance infectivity of some viruses by digesting the peritrophic

membrane. Enhancin is found in a few lepidopteran NPVs (e.g., Ld-, Cf-, and MacoNPV) and GVs (e.g., Ag-, As-, Tn-, XcGVs). Another group consists of stromelysin1-like metalloproteinases. Orthologs of this family are found in all sequenced GV genomes. The third metalloproteinase is cathepsin, which is found in most lepidopteran group I and II viruses, but is only present in three GVs genomes. It is not present in the genomes of the hymenopteran and dipteran viruses.

Midgut. The site of that baculovirus occlusion bodies are dissolved and infection is initiated. It is where food digestion takes place in lepidopteran larvae.

MNPV/SNPV, Multiple (M) or singly (S) enveloped nucleocapsids. The morphology of nucleocapsids, in which multiple or single nucleocapsids are present within an envelope. MNPVs are found in Group I and II lepidopteran NPVs and are normally not present in GVs, or hymenopteran, or dipteran NPVs. Although characteristic of viral lineages, it does not appear to be a phylogenetic trait.

Nuclear polyhedrosis virus; nucleopolyhedrovirus (NPV). The most widely distributed type of baculovirus. NPVs replicate in the nucleus and usually produce polyhedron-shaped occlusion bodies containing more than one virion.

Nudiviruses. A group of viruses pathogenic for invertebrates and related to baculoviruses. They have enveloped, rod-shaped nucleocapsids with large circular DNA genomes and share about 15 core genes with baculoviruses. However, they are not occluded and therefore are not included in the Baculoviridae.

Occlusion-derived virus (ODV). Viruses that are derived from occlusion bodies. They obtain their envelope within the nucleus. Also called OV.

P35. P35 is an inhibitor of apoptosis encoded by AcMNPV. Closely related orthologs are only found in a few Group I baculoviruses closely related to AcMNPV. A homolog has also been reported in a GV of *Choristoneura occidentalis* (ChocGV) [11] and a variant (p49) is found in a Group II NPV (SpliNPV) [12]. In addition, a p35 ortholog was identified in an entomopox virus. P35 is a substrate for an effector caspase, caspase 1, but in the process of its cleavage it irreversibly binds to and inactivates caspase 1 [13]. Subsequently p35 was found to block other categories of caspases in a similar manner - reviewed in [14]. For more information see Chapter 7.

Peritrophic membrane (PM). A tube-like membrane that separates food from the midgut epithelium. It is composed of chitin and protein.

per os (per mouth). This refers to the route of infection of insects by ingestion.

PIB, Polyhedral inclusion bodies. This refers to NPV occlusion bodies.

PIF; per os infectivity factors. Factors that are required for oral infection by ODV. An abbreviation that preceded this refers to yeast 'petite integration frequency' mutants [15].

Polydnaviruses. Polydnaviruses are not true viruses because they do not contain genetic material for their own replication. They are produced in the ovaries of parasitic wasps and the virus-like particles contain genetic material from the wasp and are injected into host lepidopteran larvae along with the wasp eggs. They are named because they contain multiple circular double-stranded DNA molecules (polydispersed DNA) encompassing up to 560 kb that encode gene products that compromise the target host immune system and other processes and are essential for the successful development of the wasp egg. There are two lineages of parasitic wasps that employ these elements; the braconid and ichneumonids and they produce bracovirus and ichnovirus polydnavirus elements, respectively. Evidence indicates that the structural proteins of the virus-like particles of the braconid lineage are related to per os infectivity factors and are derived from an integrated nudivirus-like virus [16].

Ring zone. The ring zone is a less electron dense region near the margins of nuclei and surrounding the virogenic stroma of NPV infected cells. Polyhedra initially form in this zone, but they eventually can fill the whole nucleus.

Sf-9, Sf-21. Sf-21 is a cell line that was derived from ovarian tissue from *Spodoptera frugiperda* pupae [17]. Sf-9 cells are a clonal isolate derived from Sf21 cells. Both cell lines are permissive for AcMNPV infection.

TED. In the process of characterizing AcMNPV FP mutants produced after 25 passages in *Trichoplusia ni* cells, an isolate, (FP-D), was found to contain an integrated retrotransposon that originated from the host genome. It was called transposable element D or TED [18]. This element had features of a retrovirus including long terminal repeats and was demonstrated to express gag, pol, and env-like genes that are capable of being incorporated into virus-like particles [19-21].

Tn-368. A cell line derived from *Trichoplusia ni* that is permissive for AcMNPV infection. It was derived from ovarian tissue of a virgin adult [22].

Virogenic stroma. This is an electron dense chromatin-like structure in nuclei of baculovirus infected insects. A molecular scaffold that is believed to be produced for the replication of viral genomes and the assembly of nucleocapsids.

White spot syndrome virus. See Whispovirus.

***Whispovirus*.** *Whispovirus* is the only genus in the family Nimaviridae and contain a single species, white spot syndrome virus (WSSV) that causes disease in a wide variety of crustaceans. Severe disease outbreaks of the disease are a major problem in cultured penaeid shrimp, particularly in Asia. WSSV is a non-occluded, enveloped, rod-shaped virus with a double stranded DNA genome of about 300 kb. It is highly virulent and causes major tissue damage and the infection results in white spots of calcium deposited in the shell [23]. The name *Whispovirus* is from *wh*ite *spo*t syndrome virus. Nimaviridae is from Latin nima “thread”, that refers to a flagellum-like structure protruding from the nucleocapid.

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Appendix 1 Sequenced Baculovirus genomes* from the NCBI database September 16, 2010

From: http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=10442

Baculovirus genera	Genome	Accession	Genome length	Number of orfs	Date updated
Alphabaculovirus					
***Group* I**					
Antheraea pernyi NPV	NC_008035	strain:Liaoning	126629 nt	147	5/16/06
Anticarsia gemmatalis NPV	NC_008520	isolate:AgMNPV-2D	132239 nt	152	10/21/06
Autographa californica NPV	NC_001623	-	133894 nt	156	7/16/94
Bombyx mandarina NPV	NC_012672	isolate:S1	126770 nt	141	5/14/09
Bombyx mori NPV	NC_001962	isolate:T3	128413 nt	143	1/18/96
Choristoneura fumiferana DEF MNPV	NC_005137	-	131160 nt	149	10/11/03
Choristoneura fumiferana MNPV	NC_004778	-	129593 nt	146	5/6/03
Epiphyas postvittana NPV	NC_003083	-	118584 nt	136	8/19/01
Hyphantria cunea NPV	NC_007767	-	132959 nt	148	2/2/06
Maruca vitrata MNPV	NC_008725	-	111953 nt	126	12/27/06
Orgyia pseudotsugata MNPV	NC_001875	-	131995 nt	152	3/27/97
Plutella xylostella multiple NPV	NC_008349	isolate:CL3	134417 nt	152	9/16/06
Rachiplusia ou MNPV	NC_004323	-	131526 nt	149	10/2/02
Group *II*					
Adoxophyes honmai NPV	NC_004690	strain:ADN001	113220 nt	125	4/5/03
Adoxophyes orana NPV	NC_011423	isolate:English	111724 nt	121	10/28/08
Agrotis ipsilon multiple NPV	NC_011345	strain:Illinois	155122 nt	163	10/8/08
Agrotis segetum NPV	NC_007921	-	147544 nt	153	3/27/06
Chrysodeixis chalcites NPV	NC_007151	-	149622 nt	151	6/29/05
Clanis bilineata NPV	NC_008293	isolate:DZ1	135454 nt	129	8/24/06
Ecotropis obliqua NPV	NC_008586	strain:A1	131204 nt	126	11/21/06
Euproctis pseudoconspersa NPV	NC_012639	strain:Hangzhou	141291 nt	139	4/30/09
Helicoverpa armigera NPV (2)	NC_003094	isolate:C1	130759 nt	137	8/31/01
Helicoverpa armigera NPV NNg1	NC_011354	strain:NNg1	132425 nt	143	10/10/08
Helicoverpa armigera M NPV	NC_011615	-	154196 nt	162	12/1/08
Helicoverpa armigera NPV G4	NC_002654	-	131405 nt	135	1/25/01
Helicoverpa zea SNPV	NC_003349	-	130869 nt	139	1/1/02
Leucania separata nuclear NPV	NC_008348	strain:AH1	168041 nt	169	9/16/06
Lymantria dispar MNPV	NC_001973	-	161046 nt	164	11/3/98
Lymantria xylina MNPV	NC_013953	isol.:LyxyMNPV-5	156344 nt	157	3/29/10
Mamestra configurata NPV-A (2)	NC_003529	strain:90/2	155060 nt	169	3/29/97
Mamestra configurata NPV-B	NC_004117	-	158482 nt	168	8/25/02

Orgyia leucostigma NPV	NC_010276	isolate:CFS-77	156179 nt	135	1/18/08
Spodoptera exigua MNPV	NC_002169	-	135611 nt	139	12/29/99
Spodoptera frugiperda MNPV (2)	NC_009011	isolate:3AP2	131331 nt	143	2/16/07
Spodoptera litura NPV	NC_003102	strain:G2	139342 nt	141	9/11/01
Spodoptera litura NPV II	NC_011616	-	148634 nt	147	12/1/08
Trichoplusia ni SNPV	NC_007383	-	134394 nt	145	9/7/05
Betabaculovirus					
Adoxophyes orana GV	NC_005038	-	99657 nt	119	7/15/03
Agrotis segetum GV	NC_005839	-	131680 nt	132	4/9/04
Choristoneura occidentalis GV	NC_008168	-	104710 nt	116	6/19/06
Cryptophlebia leucotreta GV	NC_005068	isolate:CV3	110907 nt	128	8/13/03
Cydia pomonella GV	NC_002816	strain:Mexican 1	123500 nt	143	4/2/01
Helicoverpa armigera GV	NC_010240	-	169794 nt	179	1/9/08
Phthorimaea operculella GV	NC_004062	-	119217 nt	130	7/1/02
Pieris rapae GV	NC_013797	isolate:Wuhan	108592 nt	120	2/11/10
Plutella xylostella GV	NC_002593	strain:K1	100999 nt	120	10/29/00
Pseudaletia unipuncta GV	NC_013772	strain:Hawaiin	176677 nt	183	1/30/10
Spodoptera litura GV	NC_009503	isolate:SlGV-K1	124121 nt	136	5/30/07
Xestia c-nigrum GV	NC_002331	-	178733 nt	181	6/7/00
Deltabaculovirus					
Culex nigripalpus NPV	NC_003084	isolate:Florida1997	108252 nt	109	8/22/01
Gammabaculovirus					
Neodiprion abietis NPV	NC_008252	-	84264 nt	93	7/24/06
Neodiprion lecontii NPV	NC_005906	-	81755 nt	89	6/17/04
Neodiprion sertifer NPV	NC_005905	-	86462 nt	90	6/17/04

* Does not include duplicate genomes – this are indicated in ()

Appendix 2

Common names of insect hosts of baculoviruses that have had their genomes sequenced

Adoxophyes honmai, the smaller tea tortrix; a pest of tea plants

Adoxophyes orana, the summer fruit tortrix moth; feeds on a wide variety of plants with a preference for apple and pear.

Agrotis segetum, the Turnip moth; can be a serious pest of root vegetables and cereals.

Agrotis ipsilon. The black cutworm

Antheraea pernyi, Chinese (Oak) Tussah Moth, Chinese Tasar Moth, also known as Temperate Tussah Moth. This is a silkworm that is raised in China for silk production.

Anticarsia gemmatalis, the velvetbean caterpillar a major pest of soybeans

Autographa californica, the alfalfa looper

Bombyx mori, the silk moth (mori from latin morus, the genus of the mulberry tree).

Bombyx mandarina. The wild silkmoth. It is thought that *B. mori* was derived from *B. mandarina.*

Choristoneura fumiferana, the spruce budworm, a forest defoliator

Choristoneura occidentalis, the Western spruce budworm

Chrysodeixis chalcites, the tomato looper (it has two distinctive gold spots on its wings)

Clanis bilineata, (no common name) Sphingidae

Cryptophlebia leucotreta, false codling moth

Culex nigripalpus (a mosquito with no common name)

Cydia pomonella, codling moth, a pest mostly of apple, pear and walnut trees

Ectropis oblique, tea looper; pest of tea

Epiphyas postvittana, light brown apple moth attacks a wide number of fruits and other plants

Euproctis pseudoconspersa. The tea tussock moth

Helicoverpa armigera, cotton bollworm, corn earworm, tobacco budworm or old world bollworm; is found in all the major crops (corn, sorghum, cotton soy, dry legumes)

Helicoverpa zea, corn earworm; cotton bollworm; tomato fruitworm

Hyphantria cunea, fall webworm; pest of many species of deciduous trees

Leucania separata, common armyworm

Lymantria dispar, gypsy moth; Deciduous forest pest

Lymantria xylina. The Casuarina Moth. A forest pest in Taiwan.

Mamestra configurata, Bertha armyworm; in Canada, it prefers canola, but also feeds on mustard, alfalfa, flax, field peas and potatoes

Maruca vitrata, bean pod borer; the soybean pod borer is known to attack legume hosts, especially cowpeas, common beans and soybeans.

Neodiprion abietis balsam fir sawfly; Forest pest

Neodiprion lecontei Redheaded pine sawfly; Forest pest

Neodiprion sertifer The European pine sawfly; Forest pest

Orgyia leucostigma The white marked tussock moth; Forest Pest

Orgyia pseudotsugata Douglas fir tussock moth; Forest pest

Pieris rapae. European Cabbage Butterfly, Imported Cabbageworm, Cabbage White.

Phthorimaea operculella The potato tuber moth; pest of potatoes in New Zealand and South Africa

Plodia interpunctella The Indian meal moth

Plutella xylostella diamond back moth; can cause severe infestations on broccoli, cabbage, canola and mustard.

Pseudaletia unipuncta. The armyworm.

Rachiplusia ou, the gray looper moth

Spodoptera exigua, beet armyworm, a pest of beets and other vegetables

Spodoptera frugiperda, the fall armyworm; a pest of corn, sorghum, and other plants of the grass family. It may also attack alfalfa, beans, peanut, potato, turnip, tomato, cabbage, cucumber, cotton, tobacco, and clove. A major source of cell lines for growth of baculoviruses

Spodoptera litura, tobacco cutworm, tobacco budworm, cotton leafworm

Trichoplusia ni, cabbage looper. A major source of cell lines for growth of baculoviruses

Xestia c-nigrum, spotted cutworm. The forewings of this species are reddish brown with distinctive patterning toward the base: a black mark rather like an angular letter c (or the Hebrew letter Nun). Also called Setaceous Hebrew Character. Note: The interpretation that I come up with is that the marking on the wings of this insect resemble Hebrew letter in a black background called nun, that resembles the letter c, hence the species name c-nigrum.