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Regulation of expression of three genes in the polyhedron envelope protein (PEP) gene region of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus (OpMNPV) was examined. These genes include open reading frame (ORF) 1 (encoding p21), ORF 2 (encoding gp16), and ORF 3 (encoding the polyhedron envelope protein). The effect of elimination of the late promoter elements of each ORF or both ORFs 1 and 2 on ORF 3 expression was examined by using an ORF 3 promoter-CAT gene fusion. The data indicated that the ORF 3 promoter was essential for the expression of the PEP. Destruction of ORF 1 caused no effect whereas destruction of ORF 2 promoter resulted in a 29% increase in CAT activity.

To characterize the role of ORF 1 and 2 in the viral life cycle and the location of the proteins in virions and infected cells, antisera were produced against these proteins. The 21 kDa protein was present in both purified budded and occluded virions as demonstrated by Western blot analysis. Immunoelectron microscopy showed that the 21 kDa protein was a capsid-associated protein in both phenotypes.

The ORF 2 gene encodes a 12 kDa protein that is N-glycosylated, migrates at a MW of 16 kDa, and is not present in budded or occluded virions. Immunoelectron microscopy indicated that gp16 is associated with lamellar-like membranous structures in close association with the

nuclear membrane. It was also found associated with envelopes of virions that had budded from the nucleus into the cytoplasm.

A gene that reportedly has a similar role to ORF 3 (polyhedron envelope protein) has been described in *Autographa californica* MNPV. This gene encodes a protein called the spheroidin-like protein (SLP) because of its sequence similarity to the spheroidin inclusion protein of the *Choristoneura biennis* entomopox virus. The gene was located, sequenced, transcriptionally mapped in OpMNPV and an antiserum was produced against a fusion protein containing most of the SLP ORF. Immunoelectron microscopy showed that the protein was concentrated in cytoplasmic inclusion bodies and was not associated with the polyhedron envelope structure in OpMNPV. It was found to be associated with polyhedra of AcMNPV, but no specific association with the polyhedron envelope was found.

The role of the PEP and the p10 protein in polyhedron morphogenesis was examined using deletion mutants of OpMNPV and immunoelectron microscopy. The p10 deletion mutant produced polyhedra with patchy and poorly attached polyhedron envelopes, suggesting p10 has a direct or indirect role in the proper formation of the polyhedron envelope. The PEP deletion mutant showed that PEP was an essential component in the formation of the polyhedron envelope. The mutant with both p10 and PEP deleted had polyhedra that showed a distinct cubic morphology. These data suggest that these two proteins may affect polyhedra morphology.

Regulation of Expression of Four Baculovirus Genes and the Immunocytochemical Characterization of Their Products.

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Christian Hans Gross

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Professor of Biochemistry and Biophysics in charge of major

Redacted for Privacy

Head of Department of Biochemistry and Biophysics

Redacted for Privacy

Dean of the Graduate School

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Typed by Christian Hans Gross

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CONTRIBUTIONS OF AUTHORS

Gregory M. Wolgamot, a Biochemistry and Biophysics undergraduate, worked with me on the contents of Chapters 3, 4 and 5.

Rebecca L. Q. Russell, a Senior Research Assistant, did the electron microscope work in Chapters 3 through 6.

Margot N. Pearson, an Assistant Professor (Senior Researcher), did the immunofluorescence microscopy in Chapters 4 and 5.

George F. Rohrmann, the principal investigator of the laboratory and my thesis advisor, provided essential guidance in data collection and the writing of the thesis.

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CHAPTER 1

Introduction

History and Taxonomy of Baculoviridae

The earliest European scientific description of a baculovirus infection was described as silkworm jaundice disease in 1808 (Nysten, 1808). In 1856, Maestri and Cornalia examined jaundiced silkworms under a microscope and observed strongly refractile crystal-like corpuscles in blood and tissues (Maestri, 1856) (Cornalia, 1856). In 1873, Bolle named these crystal-like corpuscles "polyhedrische Körnchen" or polyhedral granules. Polyhedral granules were heavier than water and insoluble in cold or boiling water, glycerol, ethanol, weak acids and other organic solvents. However, they did dissolve in dilute alkaline solutions (Bolle, 1874). In 1906, Bolle's experiments had clearly shown that the polyhedral bodies of silkworm were the infective agent of jaundice (Bolle, 1906). This disease was later referred to as polyhedral disease. The etiological agent of polyhedral disease was not identified as a virus until over a decade later.

In 1918-1919, Acqua provided convincing proof that the cause of polyhedral disease was a filterable virus (Acqua, 1918-1919). These results were not generally accepted for another decade. In 1934, cytoplasmic produced polyhedra were discovered in midgut cells of silkworms. The significance of this was not realized until 1950 when two separate and distinct virus types characterized by nuclear or cytoplasmic polyhedra were demonstrated (Smith and Wyckoff 1950). The cytoplasmic polyhedrosis viruses were later found to have double stranded, segmented RNA genomes and placed in the Reoviridae family. In the same year, dissolution of polyhedra on an electron

microscope grid revealed the rod-like shape of the nuclear polyhedrosis virus (Hughes, 1950). Therefore, they were called baculoviruses which is derived from the Greek word *baculum* meaning stick or rod.

Baculovirus research gained momentum when the first insect cell line was establilshed by Grace (1962). In 1970, the first replication of a nuclear polyhedrosis in an established cell line was acheived (Goodwin et al., 1970). During this period virus purification and cell culture techniques improved and prepared the way for investigations into the molecular biology of baculoviruses. Today we know that Baculoviridae is a large family of viruses, with over 600 identified (Martignoni and Iwai, 1986). The viruses are pathogenic predominantly for the insect order Lepidoptera (butterflies and moths), but have also been identified in the orders Diptera, Hymenoptera, Trichoptera, and Coleoptera (Adams and Bonami, 1992). Baculoviruses have also been identified in the order Crustacea. Members of the Baculoviridae are characterized by enveloped, rod-shaped virions (approximately 40 X 400 nm) that contain a circular double-stranded DNA genome ranging from 88 to 165 kb. The Baculoviridae has only one genus called baculovirus. The baculovirus genus is subdivided into two subgenera based morphological properties. Subgenus A, the nuclear polyhedrosis viruses (NPVs), usually have many virions occluded in a polyhedral occlusion body. There are two NPV morphotypes. The single-nucleocapsid NPVs (SNPV) have only one nucleocapsid in each envelope while the multinucleocapsids NPV (MNPV) have several. NPV occlusion bodies range in size from 1 to 15 mM and are predominantly composed of a single protein named polyhedrin (Rohrmann, 1986). Subgenus B, the granulosis viruses, consist of occlusion bodies between 0.25 to 0.5 mM in size, that are predominantly composed of a single protein called granulin which is related to polyhedrin (Rohrmann, 1986). A single nucleocapsid is enveloped and packaged in each occlusion body.

Baculovirus Infection Cycle

My thesis research has focused on increasing our knowledge of the regulation and immunocytochemical localization of nuclear polyhedrosis virus (NPV) structural proteins. Therefore, the introduction will now focus on NPV's. The molecular biology of two related NPVs, *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) and *Autographa californica* (AcMNPV) have been studied in most detail. The OpMNPV is pathogenic for larvae of the Douglas-fir tussock moth and AcMNPV is pathogenic for larvae of the Alfalfa looper moth. These viruses have recently received considerable attention because of their potential for use as insecticides. In addition, AcMNPV is widely used as a vector for the introduction and expression of foreign genes in insects and insect cell lines (Luckow and Summers, 1988; Miller, 1988).

The infection cycle of the NPV's is complex and involves the production of two structurally distinct virion phenotypes. The polyhedra-derived virus phenotype (PDV) is the occluded form of the virus and is responsible for the spread of the virus between insects. The second phenotype is the nonoccluded budded virus (BV) which spreads the infection systemically to other cells within the insect host. The infection cycle begins with the ingestion of polyhedra that dissolve and release the PDV when they encounter the alkaline environment of the insect midgut (see Fig. 1.1). The PDV then fuse with both the columnar epithelial cells and regenerative cells of the insect midgut (Keddie et al., 1989) and travel to the nucleus. During this time, the insect cells undergo considerable morphological changes. The cells become rounded and the nuclei enlarge, appearing to encompass most of the cellular space. In the nucleus, the host chromatin seems to be moved to the margins, making room for the virogenic stroma which is the location of viral DNA replication. In the periphery of the virogenic stroma, nucleocapsid assembly takes place. These nucleocapsids are destined for either BV or PDV phenotype. Early in infection, BV is produced (Bradford et al., 1990) that spreads the virus infection to other cells in the insect. In the insect, infected hemocytes (blood cells) also

appear to aid in the spread of the virus to other tissues by an unknown mechanism (Keddie et al., 1989). Keddie et al. hypothesized that adsorption of infected hemocytes to the basal lamina of various organs facilitates virus penetration.

Nucleocapsids destined to become budded virus bud through the nuclear membrane and temporarily acquire both the inner and outer nuclear envelope. In Chapter 4, we show that a virally encoded 16 kDa glycoprotein is associated with the nuclear membrane and with the budded virus as it emerges from the nuclear membrane. This double-membrane is then apparently lost from the nucleocapsids as they are transported through the cytoplasm to the plasma membrane (Granados and Lawler, 1981). The nucleocapsids then acquire a permanent envelope when they bud through the cytoplasmic membrane that has been modified by at least one viral encoded glycoprotein, termed gp64. The budded virus is produced after DNA replication and late in the infection cycle (Bradford et al., 1990).

The PDV phenotype is produced in the nucleus late in infection. Nucleocapsids destined to become PDV are enveloped presumably by a *de novo* synthesis of the envelope. After envelopment, PDV are occluded in the polyhedrin matrix. Very late in infection a structure, called the polyhedron envelope, forms around the mature polyhedra. Upon death and decomposition of the insect, the polyhedra are released to the environment.

Before I the discuss structural proteins of the two viral phenotypes, I will define certain terms associated with these virions. Capsid refers to the structure that forms the rod-like structure that surrounds DNA and its associated proteins. Nucleocapsid refers to capsids that contain the nucleoprotein components. Polyhedron-derived virus (PDV) refers to the enveloped nucleocapsids of the occluded virus (Fig. 2) (Rohrmann et al., 1992). Polyhedrin matrix refers to the protein crystal that surround the PDV and is composed primarily of the protein polyhedrin. Polyhedron envelope refers to the envelope that surrounds the polyhedrin matrix. Polyhedrin matrix

and polyhedron envelope protein are referred to separately as the polyhedron specific components. Occluded virus refers to PDV and polyhedron specific components.

Structural Proteins of the Budded and Occluded Virus Phenotypes

Baculovirus structural proteins are implicated in the virus infection cycle, host specificity, virulence, and environmental stability of the virus (Kuzio, 1990; Kuzio et al., 1989; William et al., 1989). Studying baculovirus structural proteins will broaden our understanding of viral-host relationships and will also contribute to the development of safer and more effective viral insecticides.

To understand the structural complexity and genetic relationship between baculoviruses, we have undertaken a systematic approach to identify and characterize baculovirus structural proteins. To date, only eight proteins associated with nuclear polyhedrosis virus have been well-characterized in terms of both their genetic location, (Fig. 1.4) expression and the proteins physical location (Fig. 1.2). They are: the fibrillar-structure-forming protein, p10 (Leisy et al.,1986b; Quant-Russell et al., 1987; Kuzio et al., 1984); the arginine-rich DNA binding protein, p6.9 (Wilson et al., 1987; Russell and Rohrmann 1990b; Maeda et al., 1991); a capsid protein, p39 (Blissard et al., 1989; Pearson et al., 1988; Thiem and Miller, 1989; Bjornson and Rohrmann, 1992) a second capsid protein, p87 (Müller et al., 1989); an envelope protein of budded virus, gp64 (Volkman 1986; Whitford et al., 1989; Blissard and Rohrmann, 1989); the occlusion protein, polyhedrin (Leisy et al., 1986a; Hooft van Iddekinge et al., 1983); a polyhedra specific protein,p74 (Kuzio et al., 1989); and the polyhedron envelope protein, p32-34 (Gombart et al., 1989a; Gombart et al., 1989b; Whitt and Manning, 1988; Bjornson and Rohrmann, 1992b).

Common Structural Proteins.

Presently there are three structural proteins common to the BV and PDV phenotypes: the p39 and p87 capsid proteins, and the p6.9

DNA binding protein (Fig. 1.2). On Western blots, p39 and p87 antisera react with BV and PDV. (Pearson et al., 1988; Müller et al., 1990). Immunoelectron microscopy showed that p39 is a component of the nucleocapsid. The random distribution of immunogold staining over the surface of the nucleocapsid indicates that p39 is a major component of the capsid rather than a specialized end structure (Russell et al., 1991). p39 genes from AcMNPV (Thiem and Miller, 1989), OpMNPV (Pearson et al., 1988; Blissard et al., 1989), and LdMNPV (Bjornson and Rohrmann, 1992a) have been sequenced. The AcMNPV and OpMNPV p39 amino acid sequences are 59% identical, and 39% and 47% identical to the LdMNPV p39 sequence, respectively (Rohrmann, 1992). All eight cysteines are conserved among the three p39 sequences suggesting they have a structural role (Rohrmann, 1992).

The p87 reading frame encodes a protein of predicted MW of 71 kDa, which has been shown by Western blot analysis to be a component of the capsid (Müller et al., 1990). Although Western blot analysis indicates the protein has a MW of 87 kDa, neither N or O-linked glycosylation accounts for the MW difference (Müller et al., 1990). A similar gene was found on the AcMNPV genome (Lu and Carstens, 1991). Immunofluorescence microscopy showed that p87 (like p39) was concentrated in the nucleus late in OpMNPV infection (Müller et al., 1990).

The nucleocapsids of baculoviruses are rod-like in shape and are composed of rings of subunits in a stacked series, with the rings aligned perpendicular to the longitudinal axis (Burley, 1982). The nucleocapsids have unique asymmetric caps as determined by electron microscopy (Teakle, 1969; Kawamoto et al., 1977a; Fraser, 1986). Because of these differences and unique staining properties, it was suggested that the cap structures are composed of different proteins than the rods (Teakle, 1969). Due to the nucleocapsid complexity, there may be several proteins involved in the formation of the structure. In Chapter 3, using immunocytochemistry we have identified a third capsid protein associated with the BV and PDV phenotypes.

In cells, histones neutralize the electrostatic repulsions of DNA allowing packaging of the DNA into highly condensed structures. In baculoviruses, histones do not appear associated with DNA in nucleocapsids (Wilson and Miller, 1986). Instead, the viral encoded p6.9 DNA binding protein may function to neutralize the phosphodiester backbone of the viral DNA, allowing the viral genome to be packaged into the nucleocapsid (reviewed in Rohrmann, 1992).

Budded Virus Structural Proteins.

To date, the glycoprotein of 64 kDa (gp64) is the only BV specific structural protein identified. gp64 has been characterized in AcMNPV (Whitford et al., 1989) and in OpMNPV (Blissard and Rohrmann, 1989) and has 78% amino acid identity (Blissard and Rohrmann, 1989). The gp64 gene has both early and late promoters that allow continued production of the protein throughout infection (Blissard and Rohrmann, 1989). By using immunoelectron and immunofluorescence microscopy gp64 was found to localize to the cytoplasmic membrane (Volkman et al., 1984; Blissard and Rohrmann, 1989). As the BV nucleocapsid buds through and aquires the plasma membrane, gp64 becomes associated with BV as a component of its envelope. BV are thought to enter the cell by adsorptive endocytosis, a process normally involved in the uptake of molecules used in metabolism. BV are believed to bind to the cell surface, gathered into invaginations of the plasma membrane called clathrin coated pits, and which then pinch off from the membrane. As the clathrin dissociates from these vesicles, two or more may fuse forming larger sructures called endosomes. Endosomes become acidic, presumably by an ATP-driven pump, that is thought to induce the viral envelope to fuse with endosomal membrane, releasing the nucleocapsid into the cytoplasm (Volkman and Goldsmith, 1985). gp64 has a signal sequence at the N-terminus that is not present in the mature protein (Sivasubramanian, 1988). The protein is N-glycosylated (Roberts, 1989) and studies with inhibitors of N-glycosylation have shown glycoslation is required for incorporation of gp64 into virions (Roberts, 1989). Presumably, glycosylation allows gp64 to be transported to the plasma membrane.

gp64 also is acylated with palmitic acid. Acylation may function in anchoring the protein to the membranes or be responsible for membrane fusion (Roberts and Faulkner, 1989).

Occluded Virus Structural Proteins.

Polyhedrin and the polyhedron envelope protein (PEP) are two well defined structural proteins specific to the occlusion bodies. There are also three other proteins which appear to be associated with the occluded virus; p10, p74, and in *Bombyx mori* a 40 kDa protein. Polyhedrin is produced in large amounts, comprising up to 18% or more of the total alkali-soluble cellular protein in infected insects (Quant et al., 1984). Polyhedrin shows the highest conservation among the baculovirus proteins with over 80% amino acid identity among lepidopteran polyhedrins (Rohrmann, 1986). The lepidopteran polyhedrins have 50% and 40% homology to granulins and NPV hymenopteran polyhedrins, respectively (Rohrmann, 1992). The conservation of the polyhedrins and granulins indicates that they evolved from a common ancestor.

Baculoviruses are not the only viruses that embed their virions in a protein matrix. Cytoplasmic polyhedrosis viruses (*Reovirdae*) and entomopoxviruses are also occluded (Payne and Mertens, 1983; Arif, 1984; Goodwin et al., 1991). The virions of Entomopoxviruses become occluded in spherical-shaped occlusion bodies called spherules that are composed of a protein called spheroidin. Spheroidin genes have been sequenced, from *Choristoneura bennis* EPV(Yuen et al., 1990) and *Amsacta moorei* EPV (Banville et al., 1992; Hall and Moyer, 1991). These gene products show no sequence homology to each other or to baculovirus polyhedrins. These results indicate that the two spheroidin proteins and the polyhedrins may have evolved independently from each other. Recently, Vialard et al. described a AcMNPV gene product called the spheroidin-like protein (SLP) that has 39% amino acid similarity to the CbEPV spheroidin protein. The CbEPV antiserum localized the SLP to the occlusion bodies but not to PDV indicating that it is a polyhedron specific component (Vialard et

al., 1990). Vialard et al. (1990) also demonstrated by immunofluorescence microscopy that the SLP appeared to be associated with the polyhedron envelope. In Chapter 5, I determined the location, sequence, and the transcriptional map of the OpMNPV SLP gene. I also examined the location of the OpMNPV SLP and the AcMNPV SLP in the virus phenotypes and infected cells.

In baculoviruses, occlusion bodies (polyhedra) are surrounded by an electron-dense amorphous polyhedron envelope. A gene encoding a protein component of this envelope has recently been identified in OpMNPV (Gombart et al., 1989 a,b). The polyhedron envelope protein (PEP) (Fig. 1.2) was discovered by screening a λ gt11 expression library with a polyclonal antiserum produced against purified dilute alkaline solubilized PDV components of OpMNPV. Two λ clones that contained nonoverlapping insert DNAs were identified. These clones mapped to a single 32.4 kDa open reading frame (ORF) in the HindIII-M fragment (Gombart et al., 1989b). The HindIII-M fragment of OpMNPV was sequenced and contains five ORF's oriented in the same direction and expressed late in infection (Fig. 1.3) (Gombart et al., 1989a). Transcriptional mapping indicated that mRNAs from the five ORFs coterminate at a single site downstream of ORF 5 (Fig. 1.3) (Gombart et al., 1989a). The conserved late gene promoter/transcriptional start sequence (A/GTAAG) was present upstream of each ORF. The late promoter element will be discussed in greater detail in the next section. Primer extension analysis showed that the late promoter upstream of ORF 3 (the polyhedron envelope protein (PEP) gene) was not the major site of mRNA initiation for the gene (Gombart et al., 1989a). This data indicated that the use of late promoter signal (A/GTAAG) for transcriptional initiation may vary between different ORFs. In Chapter 2 of this thesis, I investigate the role of the ATAAG and the upstream ORFs in expression of the PEP gene.

A *trp*E-PEP gene fusion containing the entire PEP ORF was constructed and a monospecific antiserum was produced (Gombart et al., 1989b). Western blot analysis and immunofluorescence microscopy

demonstrated that the protein was present in the cytoplasm and the nucleus at 24 hr p.i. (Gombart et al., 1989b). Studies with polyhedra solubilized under various conditions indicated that PEP is associated with the polyhedron envelope (Gombart et al., 1989b). Immunoelectron microscopy has confirmed that the PEP is an integral component of the polyhedron envelope (Russell and Rohrmann, 1990a). The addition of a reducing agent like 2-mercaptoethanol along with SDS was required to solubilize PEP from the polyhedron envelope. The PEP solubility properties are unique from other virion proteins such as polyhedrin and p39 that are found in the supernatant when polyhedra are treated with dilute alkaline saline (DAS) and SDS. This unique solubility indicated a possible thiol linkage through cysteine residue(s) with the polyhedron envelope. The solubility properties of the PEP and reciprocal immunoblotting experiments indicated that the OpMNPV PEP gene encodes a protein which is homologous to the polyhedron envelope phosphoprotein of AcMNPV, pp34 (Gombart et al.,1989b; Whitt and Manning, 1988; van Lent et al., 1990).

In AcMNPV, the PEP gene region has been sequenced and transcriptionally mapped (Oellig et al., 1987) and shares 58% amino acid identity with OpMNPV PEP (Gombart et al., 1989a). The AcMNPV PEP has a large hydrophilic region created by 16 arginine-serine repeats, not present in OpMNPV. Arginine-serine repeats may have a significant effect on the structure of the protein. The clustering of cysteine residues in each of the hydrophilic regions in both proteins may be important in the thiol linkages of these proteins to the polyhedron envelope. The large hydrophilic region found in AcMNPV may make the cysteines more available for linkage to the polyhedron envelope. One report suggested that the polyhedron envelope was composed predominantly of carbohydrate (Minion et al., 1979). However, others have shown that a protein is also a component of the polyhedron envelope (Gombart et al., 1989a,b; Whitt and Manning, 1988). The solubility properties of PEP suggest that the protein may be covalently attached to the carbohydrate of the polyhedron envelope via a thiol-glycosidic linkage. Linkages of this type have been observed before.

For example, the erythrocyte membrane glycopeptide has a triglucosylcyteine bond (Weiss et al., 1971) and in a glycopeptide isolated from human urine, a digalactosylcysteine bond exists (Lote and Weiss, 1971). It is possible that there are additional proteins associated with the polyhedron envelope.

The precise function of the polyhedron envelope is unknown. Experimental evidence indicates that it contributes to the stability of the occlusion bodies; in a recombinant AcMNPV with the p10 gene deleted, the polyhedron envelope did not form properly. These polyhedra were more fragile to physical treatments than the wild type polyhedra (Williams et al., 1989). An association between the PEP and p10 has been demonstrated using two different size gold particles in immunoelectron microscopy (Russell et al., 1991). PEP associates on the edges of fibrillar structures that are composed of p10 (Russell et al., 1991). The role of PEP and p10 in the formation of polyhedron envelope was examined by constructing three recombinant viruses: a p10 gene deleted virus, a PEP gene deleted virus, and a virus where both genes are deleted. The effects of deleting the PEP and p10 genes were examined using immunoelectron microscopy. The results are presented in detail in Chapter 6.

p10 is one of two known very late hyperexpressed genes. The p10 genes have been characterized in AcMNPV (Kuzio et al., 1984) and in OpMNPV and have 41% amino acid identity (Leisy et al., 1986b). The p10 protein is a component of fibrillar structures that form both in the cytoplasm and in the nucleus (van der Wilk et al., 1987; Williams et al., 1989; Russell et al., 1991). Although p10 is commonly found associated with polyhedra (Quant-Russell et al., 1987), it is not clear if p10 has a structural role in the polyhedra or if p10 is simply trapped during occlusion.

The association of p74 with polyhedra is based on the observation that an AcMNPV p74 mutant in which p74 is inactivated is capable of replicating in cell culture, but occluded virus produced in cell culture are not infectious when ingested by insects (Kuzio et

al.,1989). In cell culture, this mutant produced occlusion bodies that contained nucleocapsids but had a poorly defined polyhedron envelope (Kuzio et al., 1989). This result is reminscent of the virulent p10 deletion mutant of Williams et al. (1989) where the polyhedron envelope was not properly attached to the polyhedra. Kuzio et al. (1989) speculate that the p74 protein processes and/or modifies viral proteins which are not required for permissive growth in cell culture but which are essential for pathogenesis in larvae. The p74 genes have been located downstream of the p10 gene in both AcMNPV (Kuzio et al., 1989) and OpMNPV (Leisy et al., 1986b). The p74 is not N-glycosylated (Roberts, 1989). Transcription of the p74 gene occurs late and has been mapped to the sequence TATTG that is different from the consensus late promoter ATAAG (Kuzio et al., 1989).

Nagamine et al., (1991b) have sequenced a gene that encodes a 40 kDa structural protein of the nuclear polyhedrosis virus of *Bombyx mori* (BmMNPV). A monoclonal antibody specific to the protein has been shown it to be associated only with the polyhedron derived virions by Western blot analysis (Nagamine et al., 1991a). A cellular location of the protein has not yet been determined. Because the 40 kDa protein is only present in the PDV, it may be a PDV specific component like the PDV envelope.

Regulation of Baculovirus Gene Expression

Transcriptional Classes and Levels of Regulation

There are currently three transcriptional classes defined in nuclear polyhedrosis viruses: early, late, and very late genes (Rohrmann, 1992). The NPV early genes have promoters that are recognized by host cell RNA polymerase II and require no viral factors for basal levels of transcription (Hoopes and Rohrmann, 1990; Huh and Weaver, 1990). The NPV genes transcribed after the initiation of DNA replication are classed as late genes. Late genes normally have a five base pair late promoter/transcriptional start sequence: A/G/TTAAG. These late promoters are found at variable distances upstream (15 to

174 nucleotides) from the translational start codon. The late mRNAs normally initiate at the T or A, positions 1 or 2, of the TAAG sequence. Late genes are transcribed by an α-amanitin resistant RNA polymerase (Grula et al., 1981; Fuchs et al., 1983; Huh and Weaver, 1990). The RNA polymerase may be virally encoded or a modified cellular RNA polymerase. Between the two very late hyperexpressed genes (polyhedrin and p10) and the other late genes there appears to be no difference in promoter structure although there is a higher rate of transcription initiation from these promoters, and their mRNAs are stable very late in infection. The reason(s) for these differences are unknown. The transcriptional organization of NPVs has been examined by many researchers and they have found no pattern in early and late gene distribution.

Theoretically, genes can be regulated at the level of transcription initiation or elongation, or post-transcriptionally during maturation events such as 5' cap addition, addition of 3' poly (A) residues, or splicing of the message. Post-transcriptional regulation of the genes could also be influenced by mRNA transport from the nucleus to the cytoplasm or by differential stability of mature mRNA. The expression of genes can also be regulated at the level of translation or by a post-translational mechanism.

Regulation at the transcriptional level (i.e. initiation) has been proposed to play a critical role in NPV gene expression (reviewed, Friesen and Miller, 1986). The evidence for transcriptional regulation include the following: 1) Various temporal classes of viral RNA have been identified during viral infection. 2) The rapid synthesis of very late proteins closely parallels the transcription of their RNAs over a short period late in infection. This has been interpreted to mean that transcription is the limiting factor. 3) Multiple overlapping transcripts with common 3' termini have been shown to be sequentially expressed. For example, in the multiple overlapping transcription units, the shorter transcripts are expressed early and may be turned off by the longer transcripts that are expressed late in infection. Multiple

overlapping transcription units are common in NPVs and will be discussed in the next section.

Splicing does not appear to play a critical role in the regulation of NPV genes. Only one early gene (IE-1) in NPVs is known to undergo splicing (Chisholm and Henner, 1988; Kovacs et al., 1991). To date, little is known about other regulatory mechanisms that may be used by NPVs. In Chapter 2 we have examined the overlapping transcriptional unit of the PEP gene, and show that the long 3' flanking sequence may play a role in mRNA stability.

Overlapping Transcription, Promoter Occlusion, and Polycistronic mRNA's

A common transcriptional motif in nuclear polyhedrosis viruses are overlapping transcriptional units (Gombart et al.,1989a; Russell and Rohrmann, 1990b; Oellig et al., 1987; Friesen and Miller, 1987; Mainprize et al., 1986; Friesen and Miller, 1986; Friesen and Miller, 1985; Lubbert and Doerfler, 1984). Two types have been identified. The p10 and polyhedrin RNAs expressed very late in infection have their 3' ends of the RNAs extended (reviewed, Friesen and Miller, 1986). The functional significance of the extended 3' ends is not clear. An untested hypothesis is that the 3' extended ends may increase the stability of the RNAs. A second type, involves multiple RNA transcripts starting from several ORFs upstream having a common 3' termination site. For example, a series of ORFs in the PEP gene region and p6.9 DNA binding protein gene region coterminate (Gombart et al., 1989a; Russell and Rohrmann,1990b). The regulation and functional significance of the common 3' cotermination in the PEP gene region is the subject of Chapter 2. In the PEP gene region, I also examined a possible transcriptional regulatory mechanism called promoter occlusion.

Promoter occlusion and transcriptional interference describe a situation where RNA polymerases initiating at upstream promoters interfere with the establishment and initiation of RNA polymerases on

downstream promoters. An analogy would be a car attempting to get on a busy highway. Here the strength of the downstream promoter is influenced by the upstream promoter. To determine whether promoter occlusion takes place, the upstream promoter could be destroyed by a mutation, allowing the full expression of the downstream promoter. If there is an increased rate of transcription of the mutant construct over the wild type, then promoter occlusion may play a regulatory role on the downstream promoter. Overlapping transcriptional units, in which multiple promoters transcribe in the same direction, the upstream promoters could potentially regulate downstream promoters by promoter occlusion. Promoter occlusion has been documented in other systems, the human α-globin gene (Proudfoot, 1986); *Drosophila melanogaster* alcohol dehydrogenase gene (Corbin and Maniatis, 1989); ribosomal RNA transcription (Bateman and Paule, 1988); *E. coli* (Gay et al., 1986; Hausler and Somerville, 1979) and bacteriophage lambda (Adhya and Gottesman, 1982). In Chapter 2, I examine whether promoter occlusion has a regulatory role in the polyhedron envelope protein gene region.

In NPVs, overlapping mRNAs having 3' coterminal ends have the potential to encode more than one protein since each mRNA commonly contains several open reading frames. mRNAs that contain two or more translatable open reading frames are called polycistronic mRNAs. Bacteria commonly produce polycistronic mRNAs from which multiple gene products are translated. Bacterial translational initiation takes place when the 30S ribosomal subunit binds at the AUG start codon followed by the binding of the 50S ribosomal subunit to form the translation complex. Recognition of the start codon is facilitated by the upstream Shine-Dalgarno sequence that is complementary to the 16S RNA. Bacteria are capable of translational initiation at multiple sites on a polycistronic message because the ribosomes can bind to internal sites on the mRNA.

In eukaryotes, translational initiation takes place when the 40S ribosomal subunit binds at the capped 5' end of the mRNA. The 40S ribosomal subunit requires interaction with 5' cap structure to bind

efficiently (Shatkin, 1976). The 40S subunit is unable to bind circular mRNAs (Kozak, 1979a; Konarska et al., 1981). After binding, the 40S subunit translocates to the AUG start site where the 60S ribosomal subunit joins to begin translation (Kozak 1979b; Kozak, 1980). Because the 40S subunit depends on 5' cap structure interactions, most eukaryotic mRNA's have a single open reading frame and a single translational initiation site that is usually the AUG codon in closest proximity to the 5' end (Kozak, 1978). Although polycistronic mRNA's are known to occur in eukaryotes, most are of viral origin. The Modified Scanning Model (MSM) is the most comprehensive model for the regulation of translational initiation for monocistronic and polycistronic eukaryotic mRNAs. The MSM states that the frequency that a ribosome initiates or bypasses a specific AUG depends on the sequence context surrounding the AUG. Counting the A of the AUG as +1, the most important nucleotides are found in the -3 and +4 positions, with purines favored over pyrimidines in these sites (ACCAUGG optimal sequence context) (Kozak, 1986b; Kozak, 1989). In addition, evidence suggests that efficient reinitiation of translation can occur at downstream AUG codons if translation from the upstream AUG codon is terminated at least 50 nucleotides before or after the downstream AUG (Peabody et al., 1986;). Kozak (1987) has hypothesized that ribosomes are unable to efficiently recognize translational initiation signals located very close to the 5' end of an mRNA. In simian virus (SV40) and yeast, experimentation has shown that short (6 to 21 nucleotides) 5' untranslated leader increase the translation of the downstream open reading frames (Sedman et al., 1990; van den Heuvel et al., 1989).

To date, there are several examples in eukaryotic systems in which two or more open reading frames are translated from a single polycistronic mRNA (Kozak, 1986a). In tobacco plants and maize chloroplasts there are reports that a single polycistronic mRNA can encode two or more proteins (Angenon et al., 1989; Barkan, 1988). In vesicular stomatitis virus (rhabdovirus), a bicistronic mRNA is present which produces two proteins (Herman, 1986). Cauliflower mosaic virus has been extensively studied and shown to have a polycistronic

mRNA (Dixon and Hohn, 1984; Bonneville et al., 1989; Futterer and Hohn, 1991). Papillomavirus, Epstein-Barr virus, and SV40 all are reported to have polycistronic mRNAs (Barbosa and Wettstein, 1988; Wang et al., 1987; Sedman et al.,1989; Dabrowski and Alwine, 1988). In poliovirus, the 5' end of the mRNA is not capped and terminates in pUp (Hewlett et al., 1976). An internal ribosome binding site in the 5' untranslated region is responsible for the translation of the poliovirus polycistronic mRNA (Pelletier and Sonenberg, 1988). These systems may have cis elements in their mRNAs or in the translational machinery (i.e., factors that promote reinitiation or internal initiation) that allow the translation of polycistronic messages.

Transcriptional mapping of HindIII-M fragment of OpMNPV that was mentioned earlier showed that this region contained five ORF's oriented in the same direction, expressed late in infection and coterminated at a single site downstream of ORF 5 (Fig. 1.3). The nucleotide distances between the 5' cap and the AUG of each the first 3 ORF's are: 15 nucleotides (ORF 1), 18 nucleotides (ORF 2), 45 nucleotides (ORF 3). The ORF 1 AUG sequence context is strong (GXXAUGG) whereas the ORF 2 and ORF 3 is moderate (GXXAUGA). Since the 40S ribosomal subunits protect approximately 60 bases of RNA from nucleases (Kozak, 1978), the possibility exists that 5' mRNAs from ORF's 1 and 2 may contribute to the translation of PEP. In Chapter 2, I examine whether the 5' upstream mRNA's from PEP gene region contributed to the synthesis of PEP.

mRNA Stability

When steady state mRNA levels change in a system due to manipulations of DNA sequences, a transcriptional or post-transcriptional mechanism may be involved. An example of a transcriptional mechanism could be an altered rate of transcription. An altered rate of transcription could be caused by the deletion of an enhancer element or some other cis acting element which modulates transcription initiation, elongation or termination. Examples of post-transcriptional mechanisms include splicing, transport (nucleus to

cytoplasm), or mRNA stability. Manipulation of sequences involved in splicing or transport could alter the steady state mRNA levels. The deletion of a specific sequence or reduction in the length of an mRNA could cause difference in steady state mRNA levels by affecting its stability. To prove, for example, that mRNA stability is a regulatory mechanism for a given mRNA transcript, other transcriptional and post-transcriptional mechanisms must be disproved.

In cells, differential mRNA stability is an important regulatory mechanism. Cells are known to differentially regulate the turnover of mRNAs, such that some mRNAs are more rapidly degraded than others. For example, c-*myc* mRNA has a half life of less than 15 minutes, whereas β-globin mRNA has a half life of greater than 24 hours in erythroid cells (Dani et al., 1984; Ross and Pizarro, 1983). The same mRNA may also be differentially regulated under different conditions. Several well studied examples include tubulin, histone, and transferrin receptor mRNA (Gay et al., 1989; Pandy and Marzluff, 1987; Chan et al., 1989). Tubulin mRNA stability is regulated largely by the intracellular tubulin conentration. Researchers have shown that degradation of tubulin mRNA is translation dependent (Gay et al., 1989). Two models have been proposed. In first, the interaction between the free tubulin heterodimers and the emerging tubulin polypeptide causes ribosome stalling, resulting in unprotected gaps on the mRNA that may be targets for endoribonucleases. In second model, the free tubulin/polypeptide complex activates a specific ribosome-bound nuclease. Another example is histone mRNAs, which contain a stem-loop structure in the last 30 nucleotides at the 3' end of the RNA that is necessary and sufficient for cell cycle regulation of the mRNA (Pandy and Marzluff, 1987). The transferrin receptor mediates the cellular uptake of iron by binding to transferrin, the iron-containing ligand. In the presence of iron, the levels of the transferrin receptor mRNAs are reduced. Cis acting elements have been mapped to two separate 60 nucleotide stem-loop domains in the 3' noncoding sequence (Chan et al., 1989). Gel-shift and UV-crosslinking experiments have identified a cytoplasmic RNA binding protein with specificity for these domains (Mullner et al., 1989). These three

examples have illustrated how some cis elements in the mRNA may regulate mRNA stability.

In viruses, differential mRNA stability is also an important regulatory mechanism. Efficient virus assembly requires properly timed synthesis and appropriate quantities of each structural protein. mRNA stability is a regulatory mechanism that could assure that the appropriate quanitity of a structural protein is synthesized without constitutive high level transcription of the gene. Viral infections can influence the cytoplasmic stability of both host and viral mRNAs. In herpes simplex virus (HSV), the virion host shutoff protein (vhs1) functions to degrade host mRNAs and later in infection also degrades the mRNAs of the α and β gene classes (Oroskar and Read, 1989). A vhs1 minus mutant virus showed no decline of the affected mRNA classes (Oroskar and Read, 1989). In adenovirus, 5' leader sequences are spliced onto most late messages. The late L1 mRNAs primarily have additional 5' leader sequences spliced on, called i-leader, which codes for a 13.6 kDa protein (Soloway and Shenk, 1990). Soloway and Shenk have shown that mutation of the i-leader results in a substantial increase in the half-life of the L1 message over the wild type mRNA(26 hours versus < 4 hours). When the viral mutant was co-infected with wild type virus (providing the i-leader protein in *trans*), no effect on the mutant L1 mRNAs was observed (Soloway and Shenk, 1990). The authors believe that the synthesis of i-leader protein leads to the destabilization of the L1 mRNAs by a mechanism linked to on-going translation. The authors speculate that the i-leader induced destabilization is used as a fine tuning mechanism to control levels of the other proteins encoded by the L1 mRNAs which have been shown to play a role in the assembly of virions. No sequences have been identifed in baculoviruses that play a role in mRNA stability. In Chapter 2, I provide the first experimental evidence for sequences that may play a role in mRNA stability in a nuclear polyhedrosis virus.

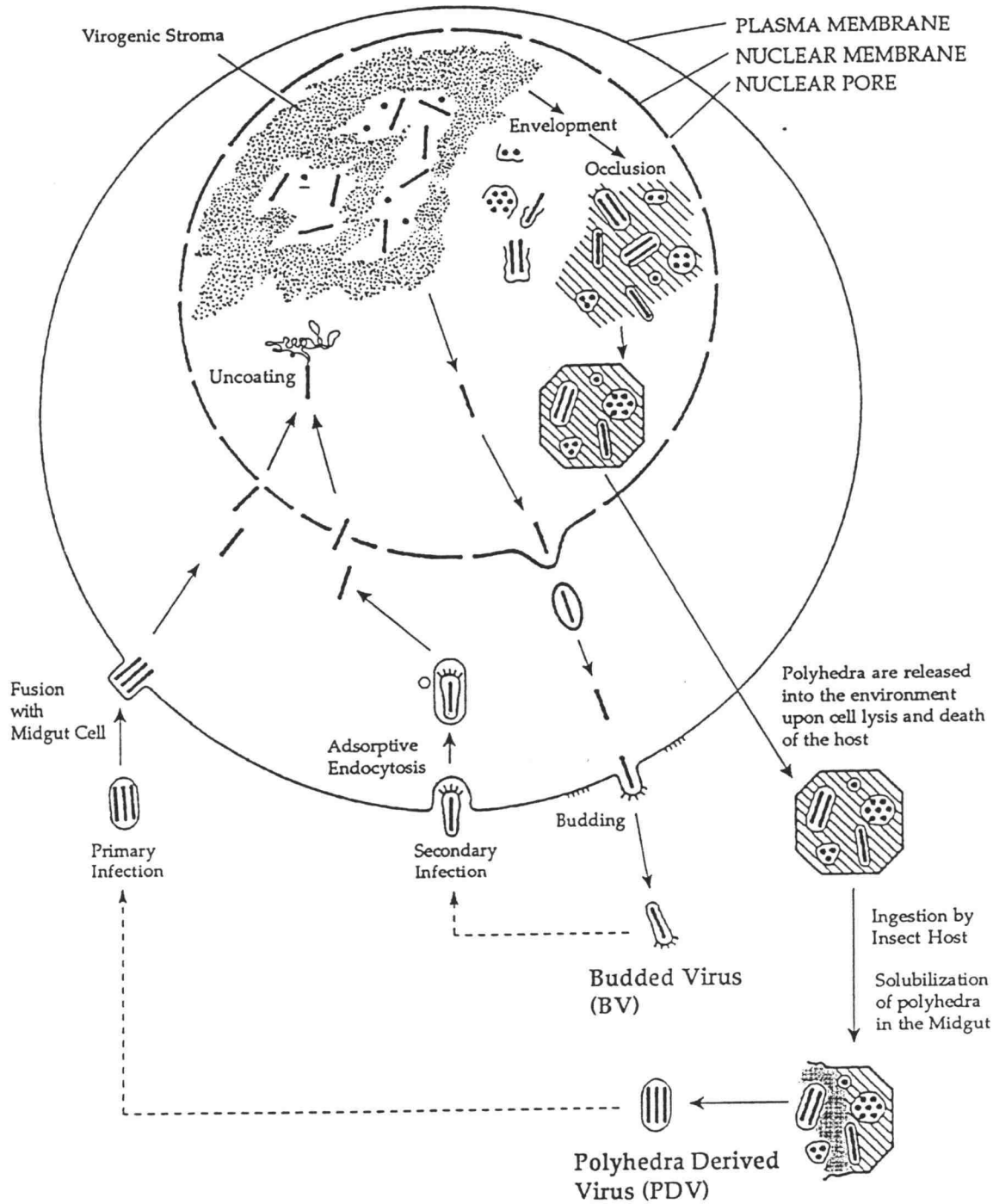


Fig. 1.1 Baculovirus infection cycle

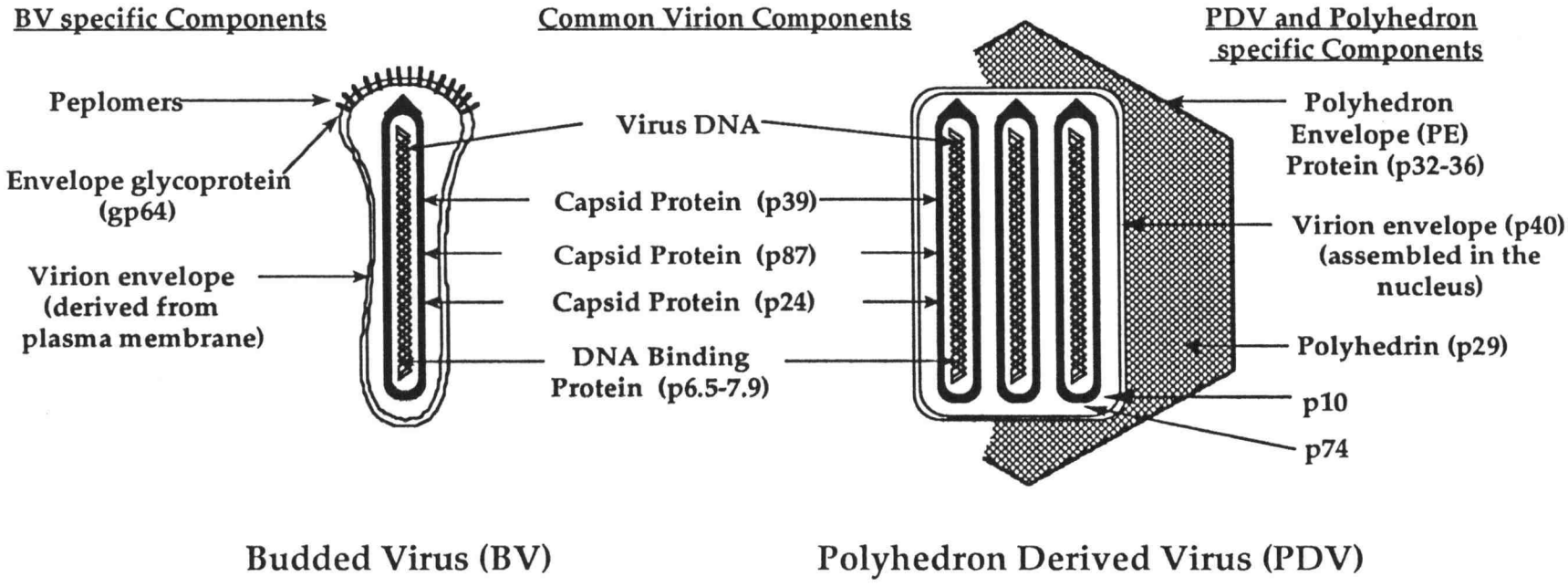


Fig. 1.2 Baculovirus phenotypes

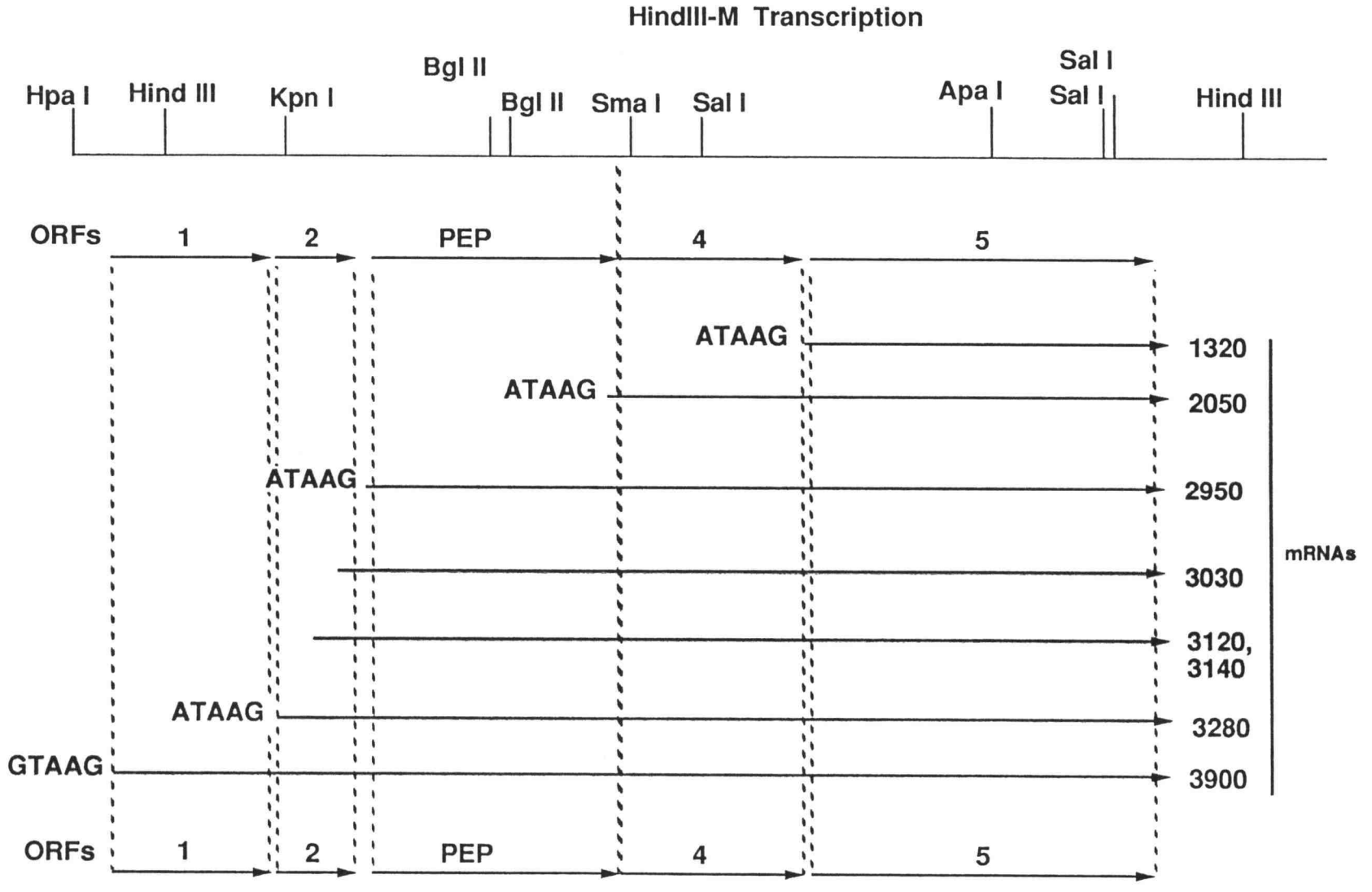


Fig. 1.3 Hind III-M transcription

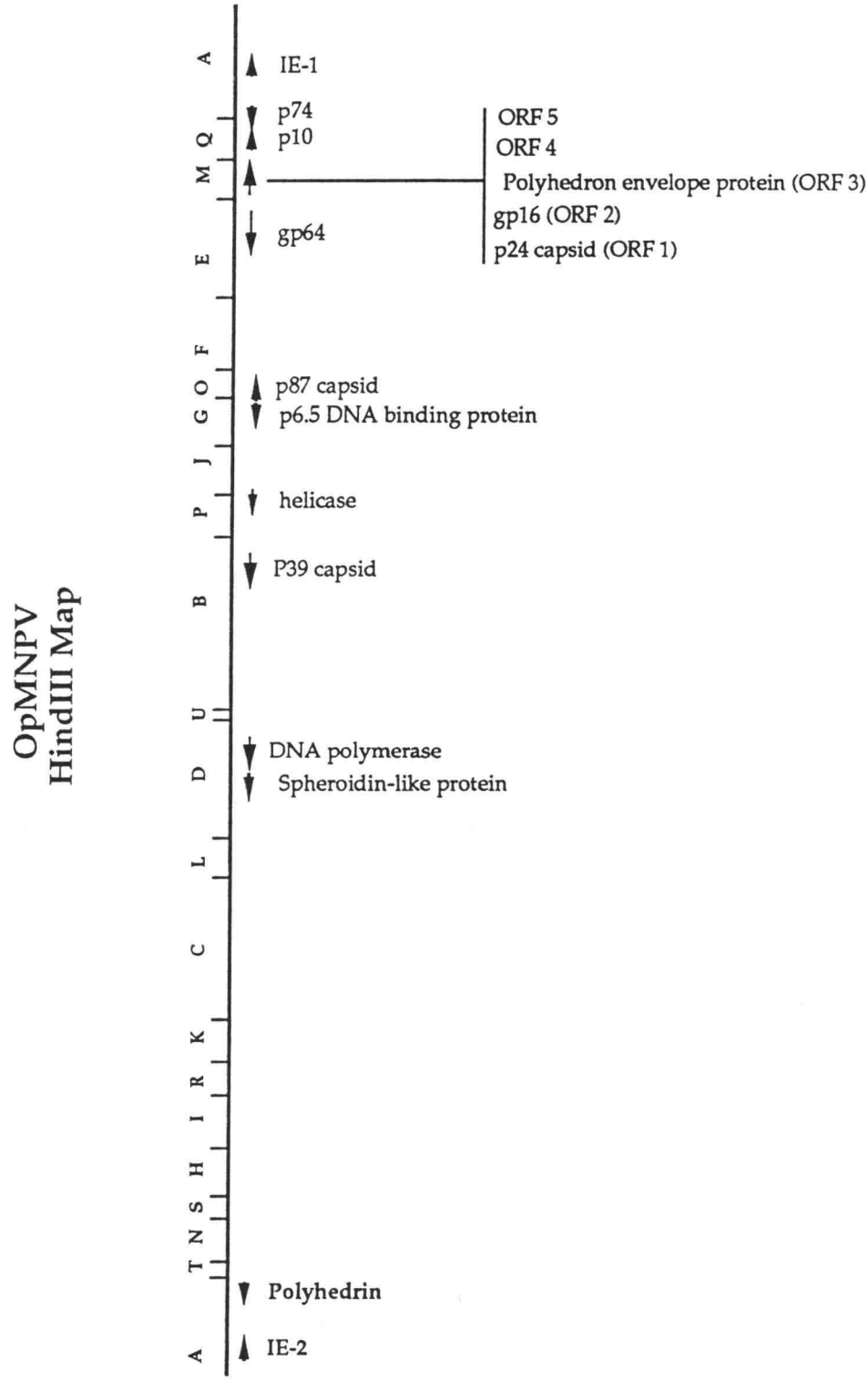


Fig. 1.4 Genomic location of the some Baculovirus genes

CHAPTER 2

Analysis of the Role of 5' and 3' Flanking Sequences on the Expression of the Baculovirus Polyhedron Envelope Protein Gene

Christian H. Gross and George F. Rohrmann

Department of Biochemistry and Biophysics
Oregon State University
Corvallis, Oregon 97331

Department of Agricultural Chemistry
Oregon State University
Corvallis, Oregon 97331

Abstract

The baculovirus polyhedron envelope protein gene is the third in a series of five open reading frames (ORFs1-5) oriented in the same direction. Individual mRNAs initiate at conserved late gene promoter/mRNA start site (A/GTAAG) elements located upstream of each ORF and the mRNAs coterminate after the fifth ORF. To examine the influence of the upstream promoter elements and the extensive 3' flanking sequence on the expression of the polyhedron envelope protein gene, the region was cloned into a phagemid vector and a chloroamphenicol acetyl transferase (CAT) reporter gene was inserted downstream of the translational start site of the polyhedron envelope protein gene. A set of clones were then constructed in which individual or combinations of the late promoter elements of ORFs 1, 2, and 3 were destroyed. These plasmid constructs were transiently assayed for CAT activity in *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus infected *Lymantria dispar* cells. Inactivation of the late promoter element immediately 5' of the polyhedron envelope protein gene led to a 97% decrease in CAT expression. Destruction of the ORF 2 promoter resulted in a 29% increase in the CAT expression. In contrast, inactivation of the ORF 1 promoter resulted in no increase in CAT expression. A variety of deletions in the 3' flanking sequence of the polyhedron envelope protein gene demonstrated that this region greatly influenced both CAT activity and steady state levels of CAT mRNA.

Introduction

Nuclear polyhedrosis viruses (NPVs) are members of the *Baculoviridae*, which are a family of viruses pathogenic for arthropods, primarily insects of the orders Lepidoptera, Hymenoptera, and Diptera. The large (88-160 kb) double-stranded supercoiled DNA genomes of NPVs replicate in the host cell nucleus. NPVs are characterized by a complex infection cycle that involves the production of two structurally distinct virion phenotypes. The budded virus (BV) phenotype, which is responsible for systemic infection within the insect host and between tissue culture cells, consists of nucleocapsids that acquire an envelope when they bud through the cytoplasmic membrane which has been modified by a virally encoded protein(s). In contrast, the polyhedra-derived virus (PDV) phenotype, acquire a *de novo*-assembled envelope within the nucleus and become occluded in polyhedron-shaped crystals called polyhera. Polyhedra are surrounded by another structure called the polyhedron envelope or polyhedron calyx and are stable in the environment until ingested by insect larvae.

A protein component of the baculovirus polyhedron envelope (calyx) was originally identified in the *Autographa californica* multicapsid NPV (Whitt and Manning, 1988) and subsequently the gene encoding this protein was identified and sequenced in both the *Orgyia pseudotsugata* multicapsid NPV (OpMNPV) (Gombart et al., 1989a) and AcMNPV (Gombart et al., 1989a; Oellig et al., 1987). These investigations indicated that in both OpMNPV and AcMNPV, the polyhedron envelope protein (PEP) gene is located as the third of five contiguous ORFs oriented in the same direction. Transcriptional mapping of the region surrounding the PEP indicates that each ORF initiates from within a consensus late promoter/transcription start sequence (G/ATAAG) which results in an overlapping set of mRNAs that coterminate downstream of the fifth ORF (Gombart et al., 1989b; Oellig et al., 1987). The overlapping transcriptional pattern seen in the PEP gene region is common in NPV's and has also been reported for four ORFs including the p6.9 DNA binding protein gene (Russell and Rohrmann, 1990b) and a variety of other regions of the AcMNPV

genome (Lubbert and Doerfler, 1984; Friesen and Miller, 1985; Mainprize et al., 1986). Although this pattern of transcriptional organization is likely to be of major importance to the virus, the influence of such an arrangment on gene expression has not been examined. In this report, we describe investigations employing a series of PEP gene promoter-CAT constructs that were designed to determine the influence that transcription from the upstream ORFs (ORFs 1 and 2) and the 3' flanking sequences has on the expression of the PEP gene.

Materials and Methods

Construction of promoter-CAT fusions

To construct PEP gene promoter-reporter fusions, we used a pBlueScribe plasmid (pBS(-), Stratagene) containing the 3.3 kbp BamHI-Hind III fragment of OpMNPV that contains the 5' region of the ORF 1 gene (Blissard and Rohrmann, 1991). This plasmid was digested with Sst I and Hpa I to remove upstream sequences, blunt-ended with S1 nuclease to allow religation of the plasmid which now contained only 240 bp of the ORF 1 5' flanking sequence. Into this plasmid the OpMNPV Hind III M fragment (Gombart et al.,1989a) was cloned in the proper orientation and the plasmid was named p1,2,PEP and contains the complete promoter regions, reading frames and intact polyadenylation signal for ORFs 1-5. A unique Bam HI site was constructed in the p1,2,PEP plasmid by site-directed mutagenesis (Kunkel et al., 1987) one codon after the translational start site of the PEP gene. This converted the native sequence **ATG**ACG**CC** to **ATG**GATCC using an oligonucleotide with the following sequence:5'-TACGTTGTTGTTGGGATCCATATTGTGTGATAT-3'. The modified p1,2,PEP plasmid was digested with Bam HI, and a 797 bp Bam HI fragment containing the CAT ORF (Mackett et al., 1984) was inserted resulting in a functional PEP promoter-CAT gene fusion (p1,2,PEPCAT) (Fig. 2.1a). A p1,2,PEP plasmid containing the CAT ORF in the opposite orientation was also constructed. The functional p1,2,PEPCAT plasmid was used in site-directed mutagenesis to create a series of plasmids with mutations in the late promoter element of the first, second, and third (polyhedron envelope protein) open reading frames. A sequence containing the late promoter element of ORF 1 (T**GTAAG**) was changed to a Bcl1 site (T**GATCA**). The promoter of ORF 2 (**ATAAG**T) was mutated to a Bgl II site (**AGATCT**). The polyhedron envelope protein promoter (**ATAAG**) was changed to (**ATGAA**) (Fig. 2.2). An additional T four bases upstream of the promoter was changed to an A creating a Xho I site. The sequence of the oligonucleotides used for these changes were ORF 1 (5'-CATGTCAATGTCCGTTGATCATTAG GGCGGAGAAAG-3'); ORF 2 (5'-GGCTAAAATTTTCAGATCTTGA

GGCACACCAGG-3'); PEP (5'-AAGTTTTGATAATAATTCATCTC TCGAGCGCGGGC-3') . To verify the mutations, all constructs were sequenced using [^{35}S] dATP (NEN) and Taq polymerase (Promega) by the dideoxy chain termination method of Sanger et al. (1977) as modified by Toneguzzo et al. (1988) for double stranded DNA. An additional clone (pPEPCAT) used in these investigations contains only the late promoter element of the PEP and was produced by cloning the Kpn-Hind III fragment of p1,2,PEPCAT in pBS(-).

3' exonuclease III deletions of the polyhedron envelope protein gene region

For functional analysis of the downstream region of the polyhedron envelope protein gene, the plasmid pPEPCAT was digested with Sst I, treated with S1 nuclease to remove Sst I site in the polylinker and then site-directed mutagenesis was used to create unique Sst I and Xba I sites at 28 and 37 nucleotides, respectively, downstream of the PEP translational start. The sequence of the oligonucleotide used to create the Sst I and Xba I sites was (5'-CGGCGTCGATCCACATCTAGACGCGAGCTCGAACATTACGTTG-3'). A series of 3' deletion subclones were generated from the modified pPEPCAT plasmid using exonuclease III (Fig. 2.5) (Henikoff, 1987). Each deletion clone was sequenced to determine the precise size of the deletion using a primer specific for the 3' end of the CAT gene.

3' Sal I-Apa I deletions of the polyhedron envelope protein gene region

In order to examine deletions specifically in the region between ORF 4 and 5 , four of exonuclease III deletion clones (see Fig. 2.6) located between the Sal I-Apa I fragment were digested with Bam HI sites which removed the CAT insert. The Bam HI sites were filled in using the Klenow fragment of DNA polymerase I, Xho I linkers were ligated to the blunt ends and the plasmid DNA was transformed into *E. coli* JM 83 cells. The Xho I-Hind III fragments (containing the deleted 3' flanking sequence) were removed from each clone and inserted into a

Xho I/Hind III digested parental pPEPCAT plasmid that had been modified by the insertion of an Xho I linker at the Sal I site in ORF 4. These constructs resulted in a nested set of deletions between the Sal I and Apa I restriction sites (Fig. 2.6).

Transfections and CAT assays

Lymantria dispar (Ld-652Y) cells were propagated in T-flasks using TNM-FH medium (Summer and Smith, 1987) supplemented with 10% fetal bovine serum. For assay of the transient expression of CAT, 1.5 x 10^6 cells were seeded on 35-mm-diameter wells of a multi-well tissue culture plate (Falcon). After allowing cells to attach, the medium was removed from each well and replaced with 800 µl of medium containing OpMNPV virus at a m.o.i. of 10. Cells were incubated at 27 °C for 4 hours and then transfected with 20 µg of plasmid DNA by calcium phosphate precipitation (Graham and Van Der Eb, 1973) as modified for insect cells (Summers and Smith, 1987). The medium was removed and replaced with a transfection buffer-DNA mixture and incubated at 27 °C. After 4 hours, the transfection mixture was replaced with fresh medium and incubated at 27 °C for 72 hours. For smaller constructs, equimolar amounts of DNA were used and supplemented with pBS(-) vector DNA to bring the total DNA to 20 µg. In addition to promoter-reporter constructs, cells were also transfected with two control plasmids: pBS(-) and pCAT, a plasmid containing the CAT ORF (Mackett et al., 1984) but no viral sequences. To assay for CAT activity, transfected cells were gently removed from the wells with a rubber policeman, pelleted (3 mins, 4000xg), resuspended in 50 µl of PBS (120 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.4)) and 5 mM EDTA, lysed by three freeze-thaw cycles, and the cell debris was pelleted at 8000xg for 3 min. CAT assays were performed on the supernatants by the two-phase fluor diffusion assay method (Neumann et al, 1987). For each CAT assay, 80 µg of protein, as determined by Bradford protein assay (Bradford, 1976), was used in a 250 µl reaction mixture containing 100 mM Tris (pH 7.8), 1 mM chloramphenicol, and 0.1 µCi [^{14}C] acetyl coenzyme A (4 mCi/mmol; New England Nuclear). Reactions were performed in

miniscintillation vials and each reaction mixture was overlaid with 5 ml of Econofluor (New England Nuclear). Acetylation of chloramphenicol was measured by direct scintillation counting at 3-6 hours after the start of the reaction. Reactions containing 0.05 and 0.1 units of CAT (Sigma) were prepared and used as positive controls to confirm the linearity of the assay at the times sampled. For each experiment, the counts per minute of acetylated chloroamphenicol obtained from extracts of cells transfected with different constructs were directly compared. To determine the degree of variability between transfections, triplicate transfections were performed for each construct.

RNA isolation, primer extension analysis, and RNase protection

For isolation of total cell RNA, six 35-mm-diameter wells of a multi-well tissue culture plate were seeded with 1.5×10^6 cells/well for each sample. Cells were infected and transfected as described above. After 48 hr, the supernatant was removed and the plates containing the cells were quick-frozen at -80 ^{o}C. To isolate total infected cell RNA, the frozen cells were scraped from the plates immediately into a 250 µl guanidinium isothiocyanate solution (4 M guanidinium isothiocyanate, 0.1 M 2-mercaptoethanol, 5 mM sodium citrate pH 7.0, and 0.5% sarcosyl) (Glisin et al, 1974). The extract produced from six wells was added to a 15 ml centrifuge tube containing 0.6 g of CsCl, sonicated for 30 sec to shear DNA, layered on a pad of 1.4 ml of 5.7 M CsCl, 0.1 M EDTA, and centrifuged at 110,000 g for 16 hr. After removing the supernatant and pad, the RNA pellets were resuspended in 1X TES (10 mM Tris pH 7.5, 5 mM EDTA, 1% SDS), immediately extracted with phenol/chloroform, and ethanol precipitated. For primer extension assays, 75 µg of each RNA sample was annealed to a 5' end-labeled oligonucleotide complementary to the 5' end of the CAT ORF and processed as described in Chapter 5. RNase protection experiment using 50 µg of RNA were performed as described by Gross et al. (1987). Primer extension and RNase protection products were electrophoresed on 8% polyacrylamide 7 M urea gels.

Results

Analysis of the influence of the transcription of upstream ORFs on the polyhedron envelope protein gene-CAT expression

In order to examine transcription of the polyhedron envelope protein (PEP) gene region, a plasmid was constructed that contained all five ORFs from this region. The ORF encoding the PEP gene (ORF 3) was altered by site-directed mutagenesis that allowed fusion of the chloroamphenicol acetyl transferase (CAT) reporter gene immediately downstream of the ORF 3 ATG (Fig. 1.1). Using site-directed mutagenesis, four mutants were produced from this parent construct in which the promoters elements of ORF 1, ORF 2, ORF 1 and 2, and ORF 3 (PEP) (Fig. 2.2, Fig. 2.3) were inactivated. A Kpn I-Hind III subclone of p1,2,PEPCAT was also constructed that contains only the PEP late promoter element (Fig. 2.3). To determine the influence of these promoter mutations on the expression of the PEP-CAT gene, plasmid DNAs (in triplicate) were transfected into OpMNPV infected *Lymantria dispar* cells. After 72 hr p.i., extracts of the cells were assayed for CAT activity. It was found that inactivation of the ORF 3 (PEP) promoter element resulted in a 97% reduction in CAT activity (Fig. 2.3, number 1 versus 6). Inactivation of the ORF 1 promoter element showed little effect on CAT expression (Fig. 2.3, number 2). When the ORF 2, or ORF 1+2 promoter elements were inactivated, an increase in CAT expression of over 15-25% was evident (numbers 3 and 4). Deletion of both ORF 1 and 2 promoter elements, a 35% elevation of CAT expression was detected (number 5). Controls included the parental constructs with CAT in the reverse orientation, the pCAT plasmid alone, pBS, or infected cells that were not transfected (Fig. 2.3, numbers 7-11) and showed no activity.

To determine if the PEP-CAT transcripts from the transfected plasmid constructs initiated at the same position as the PEP gene *in vivo*, total cell RNA was isolated, annealed to a CAT-specific oligonucleotide, and extended with reverse transcriptase. All constructs, with the exception of the plasmid that contained the PEP

promoter mutation, initiated at the T of the ATAAG (Fig. 2.4, lanes 1-5) indicating that mutation of upstream promoter elements did not influence the mRNA initiation site of the PEP-CAT gene construct. Total cell RNA from mock and infected, cells that were not transfected were also isolated for use as controls. They showed no primer extension products (Fig. 2.4, lanes 7 and 8) indicating the primer extension products in the other lanes are specific for CAT RNA transcribed from the transfected plasmids.

Analysis of the influence of the downstream untranslated sequence on the polyhedron envelope protein gene-CAT expression

To examine the influence of the 3' flanking sequence on PEP-CAT gene expression, site-directed mutagensis was used to insert a unique Sst I and Xba I restriction sites at 28 and 37 nucleotides, respectively, downstream of the PEP translational start (ATG). A nested set of unidirectional deletions were constructed and selected deletions were isolated (Fig. 2.5). Plasmid DNA was transfected into OpMNPV infected *Lymantria dispar* cells and assayed for CAT activity. For each construct, three independent transfection experiments were performed. Although deletion 2 (373 bp) showed almost parental levels of CAT activity, deletions of 43, 755, 914, and 1261 bp showed lower levels of activity (58-74%) (Fig. 2.5, numbers 1,3-5) relative to the parent construct. There was a 40% reduction in activity between the 1261 and 1645 bp deletions (number 6). Deletion of 2164 bp resulted in a construct that contained much of ORF 5 and the intact polyadenylation signal. This plasmid showed background levels of CAT activity (Fig. 2.5, number 8). Controls included the parental construct with CAT in reverse orientation, pCAT alone, pBS plasmid, and extracts from infected cells that were not transfected. These controls showed no significant levels of CAT expression (Fig. 2.5, numbers 10-13).

Because there was a major reduction (40%) in CAT activity from the constructs with deletions between the Sal I and Apa I sites (nt 1261 to nt 1615), this region was examined in greater detail. Deletions

through the Sal I-Apa I region were constructed (Fig. 2.6), transfected into infected cells and the levels of CAT activity was measured. Deletion of 54 and 412 bp downstream of the Sal I site resulted in 33 and 46% reduction, respectively, in CAT activity (Fig. 2.6, numbers 1 and 2). More extensive deletions (733 and 957 bp) resulted in the progressive reduction in CAT expression levels (numbers 3 and 4). These experiments suggested that certain regions of the 3' flanking sequence influenced CAT expression levels and as the deletions became more extensive a progressive declined in CAT activity was seen. To determine if this decline was sequence dependent, Sst I-Sma I and Sma I-Apa I deletions were "repaired" by the adding of filler DNA that were comprised of baculovirus DNA sequences lacking regulatory elements, e.g. a 750 bp Sau 3A fragment from the AcMNPV DNA polymerase ORF (Tomalski et al., 1988) was used to replace (in both orientation) the 914 bp Sst I-Sma I deletion. The Sma I-Apa I deletion was "repaired" by adding in both orientations a 1418 bp sequence (Bgl II to Sst I fragment) from the OpMNPV p87 ORF (Müller et al., 1990) and the 750 bp AcMNPV DNA polymerase fragment fused to a 515 bp sequence (the first Sal I fragment) from the OpMNPV p39 ORF (Blissard et al., 1989) was also placed in the Sma I-Apa I deletion site. The Sst I-Sma I deletion replaced by the DNA polymerase fragment in either orientation showed no increase in CAT activity was observed (data not shown, see appendix A2.3). In the Sma I-Apa I deletion replaced with the p87 ORF in both orientations showed an increase from 60% to 61% of parental CAT activity was observed (data not shown). In contrast, in the Sma I-Apa I deletion repaired with p39 ORF and DNA polymerase fragment, no increase in CAT activity was observed (data not shown, see appendix A2.3). These results suggested that loss in CAT activity was influenced by a specific sequence rather than the length of the 3' flanking sequence.

RNAse protection assays

As the 3' flanking sequence was progressively deleted, a decline in CAT activity was observed. To determine if the decline in CAT activity was regulated at the translational or transcriptional level,

RNase protection assays were conducted to determine whether steady state levels of CAT RNA had declined in constructs with low levels of CAT activity. From each construct, 50 μg total infected cell RNA was assayed. The RNAs from infected cell and synthetic RNA standards produced from T7 promoter or yeast RNA (digestion control) were hybridized to a complementary radiolabeled CAT RNA probe (Fig. 2.7a). After hybridization, nonhybridized sequences were removed by RNase digestion and the protected fragments resolved by gel electrophoresis. RNA isolated from cells transfected with plasmids that showed high levels of CAT activity, showed substantial levels of protected CAT-specific RNA (parent plasmid and lanes 1, 2, 4, Fig. 2.7b). However, CAT-specific RNA from constructs that showed greatly reduced levels of CAT activity, were present at greatly reduced levels (lanes 3,5-8, and 1-4 on the right Fig. 2.7b). These result indicated that deletions in the 3' flanking sequence either resulted in a reduction in the level of transcription of the PEP-CAT gene or, more likely, resulted in unstable transcripts that were readily degraded.

Discussion

Baculovirus genes are often arranged in the same orientation and transcribed such that a series of overlapping transcripts are produced each from an independent promoter but terminating at the same location. In this study we examined the influence of 5' and 3' regions on the expression of the polyhedron envelope protein gene region which is the third of five ORFs demonstrating this transcriptional pattern. It has been suggested that a transcriptional regulatory mechanism called promoter occlusion might be involved in the regulation of these regions (Friesen and Miller, 1985). In promoter occlusion, an RNA polymerase complex that is initated at an upstream promoter interferes with the transcriptional initiation of downstream promoters. This presumably is caused by blocking the formation of transcriptional complexes as the polymerase complex moves through the downstream sequence. Promoter occlusion has been described in other systems including the human α-globin gene (Proudfoot, 1986); *Drosophila melanogaster* alcohol dehydrogenase gene (Corbin and Maniatis, 1989); avian retroviruses (Cullen et al., 1984); ribosomal RNA transcription (Bateman and Paule, 1988); *E. coli* (Gay et al., 1986; Hausler and Sommerville, 1979) and in bacteriophage lambda (Adhya and Gottesman, 1982). In these systems, promoter occlusion has been demonstrated to profoundly affect the expression of downstream genes resulting in reduction in expression levels of 3 to 30 fold. By constructing a series of upstream promoter mutations, we have been able to examine the influence of upstream transcriptional initiation on the expression of PEP-CAT gene construct. These investigations indicated that whereas mutation of the the most distant promoter had no affect on expression levels of PEP-CAT expression, mutation of the promoter immediately upstream or deletion of the two upstream promoters caused a modest (at most 35%) increase in the PEP-CAT expression level. Therefore, if initiation from upstream baculovirus promoter elements occluded downstream promoters, it occurs at a level far lower than the other examples of promoter occlusion cited above. Factors reported to influence low levels of promoter occlusion are a large distance to the the upstream promoter or a low natural level

of transcription through the downstream promoter. For example, in lambda, promoter occlusion of the downstream promoter was inversely proportional to the distance between the two promoters and was also shown to be dependent on the strength of the upstream promoter (Adhya and Gottesman, 1982).

We also demonstrated that the PEP-CAT gene expression was regulated almost exclusively by the PEP gene late promoter element. When the PEP promoter was inactivated, CAT expression was almost completely eliminated even though copies of the PEP-CAT ORF were present on the mRNAs originating from the ORF 1 and ORF 2 promoter elements. Therefore, these overlapping mRNAs do not appear to function as polycistronic messages. Although evidence suggests that efficient reinitiation of translation occurs on eukaryotic polycistronic messages if the spacing between the termination codon and the next ATG was 50 nt or less (Peabody et al., 1986). Spacing between ORF 2 termination and PEP-CAT translational start (ATG) was 42 nt, yet no evidence of substantial levels of reinitiation of translation was evident. In addition, it has been shown in SV40 and yeast that short (6 to 21 nt) 5' untranslated leader sequences increased the translation of the downstream ORFs on polycistronic mRNA (Sedman et al., 1990; van den Heuvel et al., 1989). This may be due to the 40S ribosomal subunit, which protects approximately 60 bases of mRNA from digestion with nucleases (Kozak and Shatkin, 1978) may be sterically hindered by short 5' leader sequences and therefore efficiency of initiation at the 5' ATG is reduced. Although the untranslated leaders sequences for ORFs 1 (15 nt) and ORF 2 (18 nt) are short, the phenomenon did not appear to occur in this system.

Evidence from these investigations suggests a compelling role for the 3' flanking sequence in regulating the levels of expression of the PEP gene. A pattern of decline in CAT activity was observed in plasmids from which progressively larger segments of the flanking sequence were deleted. Using RNase protection analysis, we were unable to detect parental mRNA levels in cells transfected with 3' deletion constructs that showed low levels of CAT activity. These data

indicate that these sequences are likely to influence the rate of transcription or the stability of the mRNA. However, post-transcriptional events in which the reduced 3' flanking sequences causes a reduction in stability in the nucleus, inefficient nucleocytoplasmic transport, or inhibition of polyadenylation can not be ruled out. There are a number of examples in which 3' untranslated sequences influence mRNA levels. In contrast to this report, in human papillomavirus, a negative regulatory element located in the 3' end of L1 RNA that when deleted has been shown to increase mRNA stability (Kennedy et al., 1991). In the bovine papillomavirus late 3' untranslated region, an element has been identified that if deleted increases cytoplasmic polyadenylated RNA. This element acts before the mature mRNA reaches the cytoplasm (Furth and Baker, 1991). These data suggested that the element effects nuclear stability and/or inhibition of polyadenylation or nuclear transport.

Fig. 2.1 The polyhedron envelope protein gene region showing the location of the CAT reporter gene. A restriction map of the polyhedron envelope protein gene region is shown at the top of the diagram. A Bam HI site was mutated downstream of the PEP gene ATG for insertion of the CAT reporter gene. The location and direction of ORFs 1-5 (ORF 3 is PEP) and the location of the CAT gene is shown directly below the restriction map. A transcriptional map of the polyhedron envelope protein gene region previously determined by Gombart et al. (1989b) is shown at the bottom of the diagram. The arrows representing each transcript is preceded by a late promoter sequence. Abbreviations: Apa, Apa I; Bam, Bam HI; Bgl, Bgl II; Hind, Hind III; Hpa, Hpa I; Kpn, Kpn I; Sma, Sma I; and Sal, Sal I.

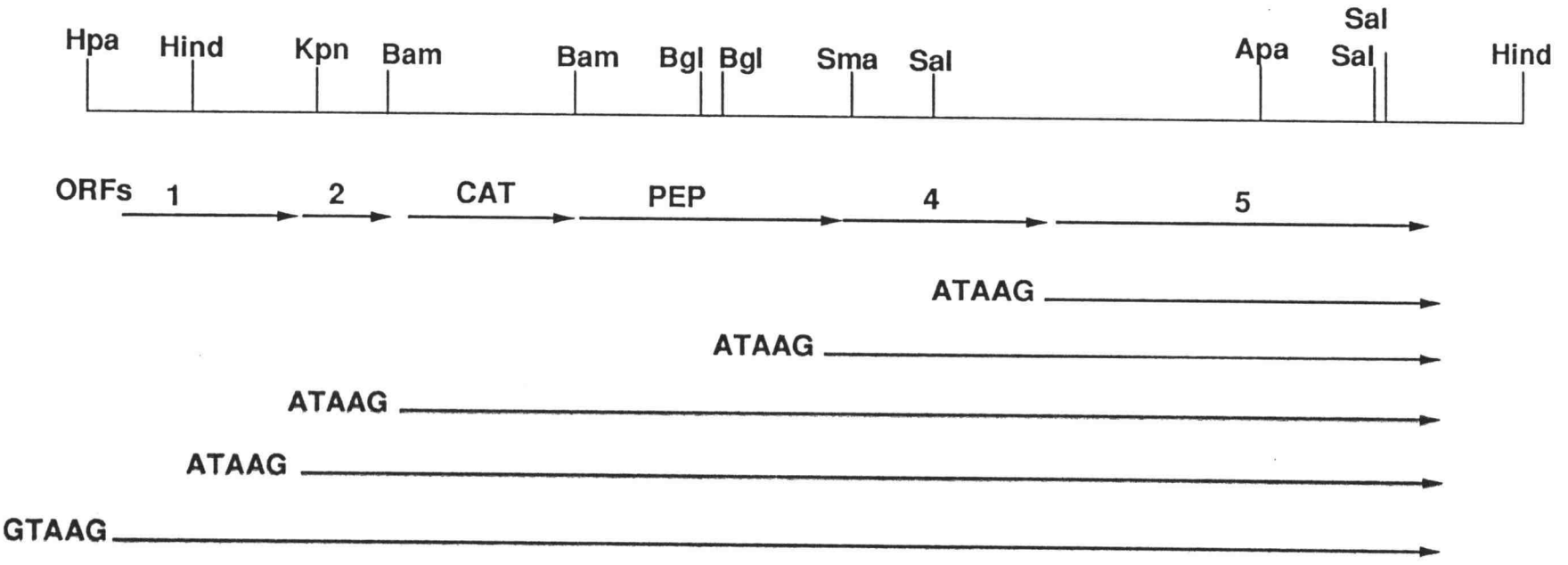

Hpa
Hind
Kpn
Bam
Bam
Bgl
Bgl
Sma
Sal
Apa
Sal
Sal
Hind
ORFs
1
2
CAT
PEP
4
5
ATAAG
ATAAG
ATAAG
ATAAG
GTAAG

Fig. 2.1

Fig. 2.2 Mutation of the ORF 1, 2 and 3 promoter sequences. The line below each sequence indicates the changes that were made in the promoter sequence. The late promoter elements and mutations are underlined. The translational start (ATG) of each ORF is also underlined and the restriction site created to screen for the mutations are indicated.

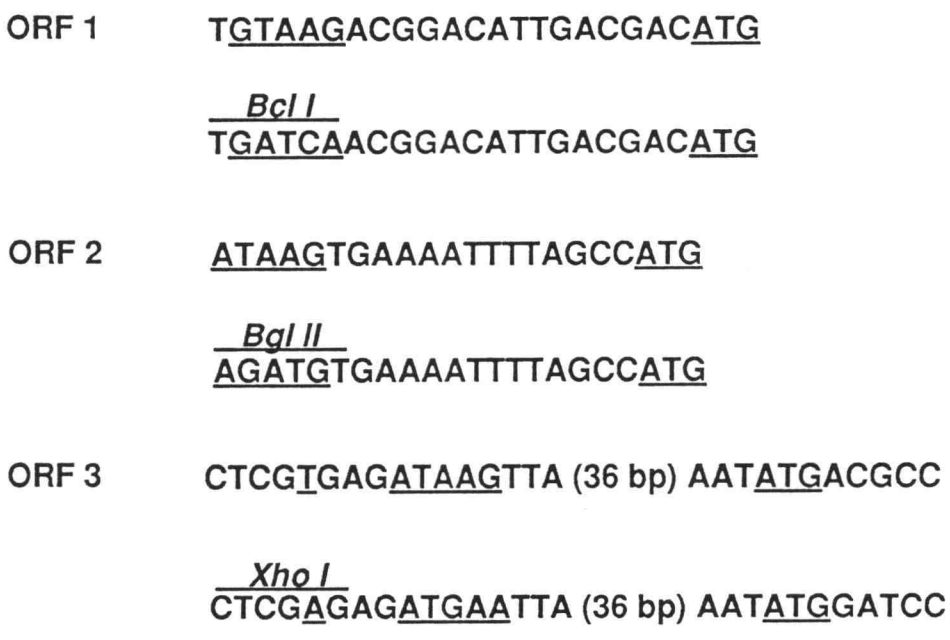

ORF 1
TGTAAGACGGACATTGACGACATG
Bcl I
TGATCAACGGACATTGACGACATG
ORF 2
ATAAGTGAAAATTTTAGCCATG
Bgl II
AGATGTGAAAATTTTAGCCATG
ORF 3
CTCGTGAGATAAGTTA (36 bp) AATATGACGCC
Xho I
CTCGAGAGATGAATTA (36 bp) AATATGGATCC

Fig. 2.2

Fig. 2.3 The effect of upstream promoter mutations on the levels of CAT expression from a polyhedron envelope promoter-CAT gene fusion. In the upper left is a map showing the three promoter elements regulating the expression of ORFs 1, 2 and CAT. Directly below this map is shown a series of schematic maps (numbered 1 through 6) indicating which of the promoter elements were inactivated. To the right of each map is a bar graph showing the levels of CAT activity corresponding to each construct. The results of a series of controls (numbered 7 through 11) are shown on the lower part of the bar graph. Controls include: the CAT reporter gene in the reverse orientation in the parent clone (see map 1, this figure) and the deletion clone (see map 5, this figure); pCAT, pBS and infected cells (IC) that were not transfected with plasmid DNA. Relative CAT activity is indicated on the X axis as counts per minute (above background) of ^{14}C-acetylated chloroamphenicol. Error bars represent the standard error detected from three transfections for each construct.

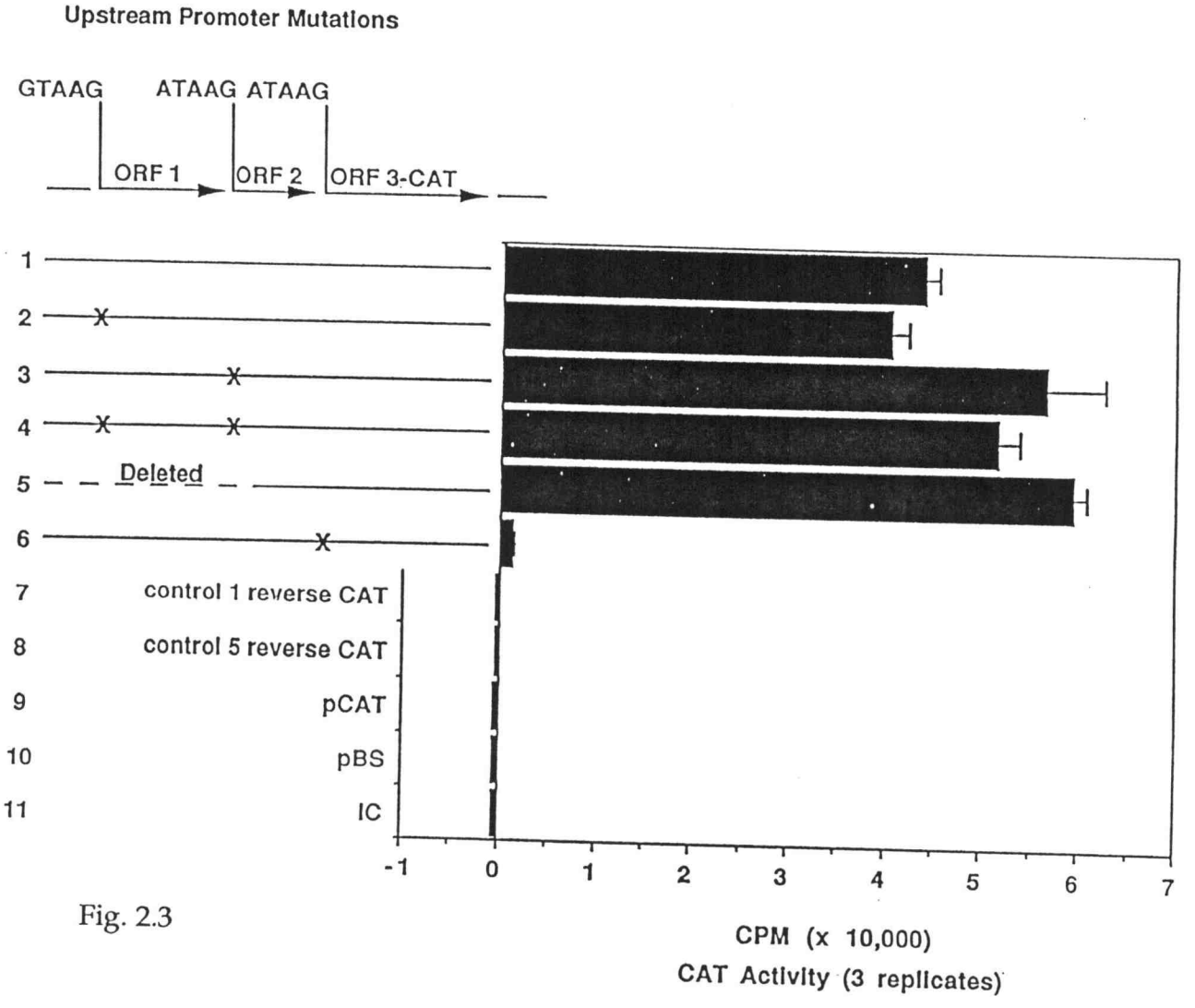

Upstream Promoter Mutations
GTAAG
ATAAG
ATAAG
ORF 1
ORF 2
ORF 3-CAT
1
2
3
4
5
Deleted
6
7
control 1 reverse CAT
8
control 5 reverse CAT
9
pCAT
10
pBS
11
IC
-1
0
1
2
3
4
5
6
7
CPM (x 10,000)
CAT Activity (3 replicates)

Fig. 2.3

Fig. 2.4 Primer extension analysis of transcripts from plasmid constructs transfected into OpMNPV infected *Lymantria dispar* cells. Plasmid constructs (Fig. 2a) were used to transfect infected Ld cells and total RNA was isolated and analyzed by primer extension analysis using a 20-nt CAT primer (5'-CGGTGGTATATCCAGTGAT-3'). Numbers above lanes indicate: 1) parental construct; 2) ORF 1 promoter mutation; 3) ORF 2 promoter mutation; 4) ORF 1 and 2 promoter mutation; 5) ORF 1 and 2 5' sequences deleted; 6) PEP promoter mutation; 7) uninfected cells; 8) infected cells. Size of primer extension products were determined by comparison to a sequencing ladder produced using the same CAT primer to sequence p1,2,PEPCAT plasmid DNA. The primer extension products for the polyhedron envelope protein late gene promoter are indicated on left by an arrow that correspond to the T in the ATAAG.

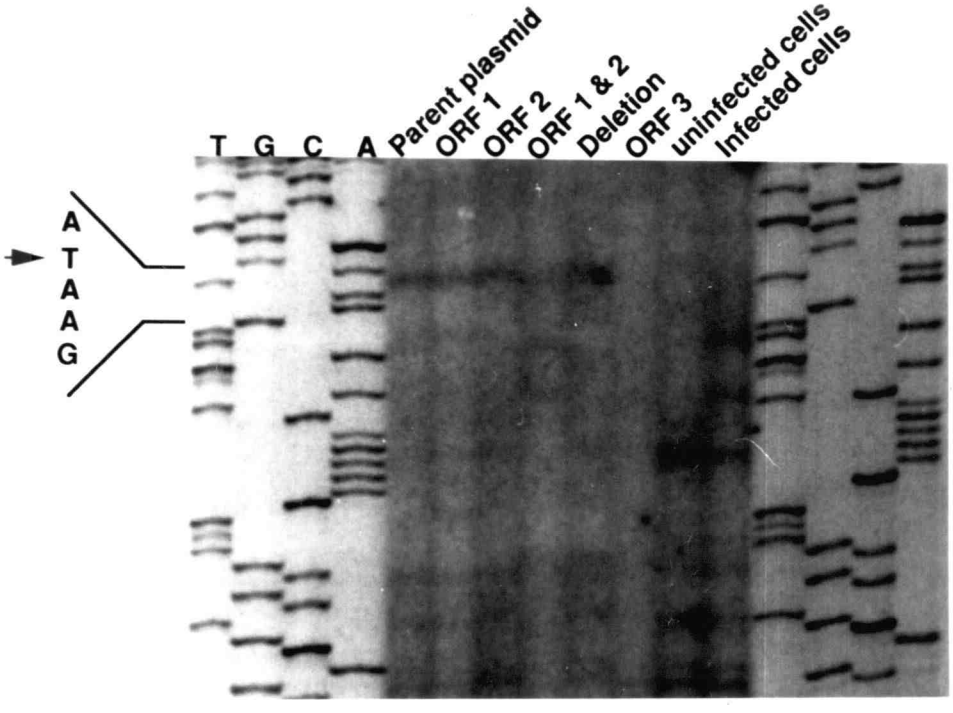

T G C A
Parent plasmid
ORF 1
ORF 2
ORF 1 & 2
Deletion
ORF 3
uninfected cells
Infected cells
A
T
A
A
G

Fig. 2.4

Fig. 2.5 The effect of deletions in the 3' flanking sequence on the levels of CAT expression from a polyhedron envelope promoter-CAT gene fusion. The left side of the figure shows the schematic representation of the location and extent of each 3' deletion mutant. Below is shown a scale indicating the position and number of base pairs deleted for each mutation of the insert DNA. To the right of the constructs is a bar graph showing the level of CAT expression from the corresponding deletion mutant. The number to the left in parentheses is the deletion size in base pairs. Controls include transfections with the wild type construct with CAT reporter gene in the reverse orientation, pCAT, pBS and infected cells (IC) that were not transfected. Relative CAT activity is indicated on the X axis as counts per minute (above background) of ^{14}C-acetylated chloroamphenicol. Error bars represent the standard error detected from three transfections.

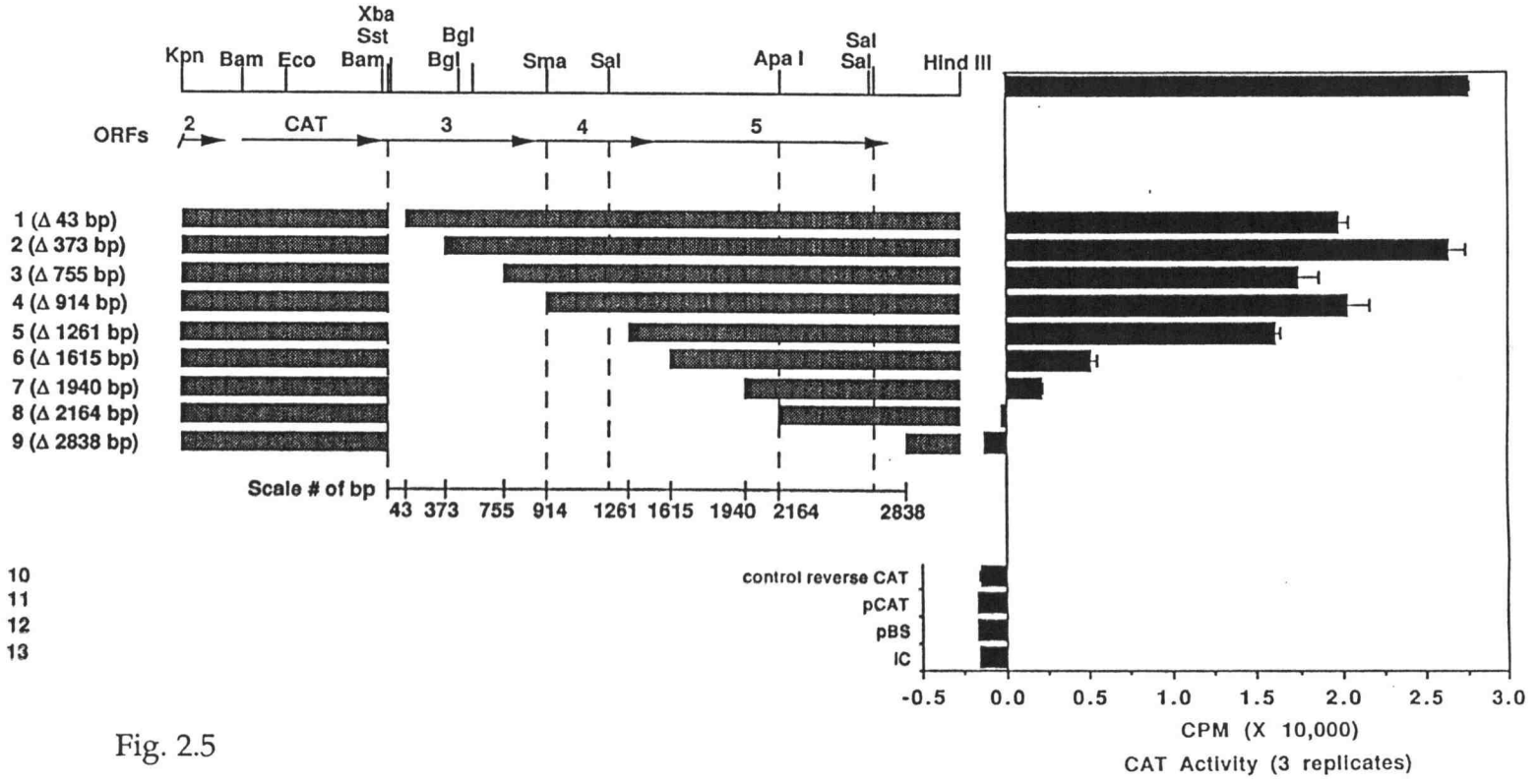

Xba
Sst
Bgl
Sal
Kpn
Bam
Eco
Bam
Bgl
Sma
Sal
Apa I
Sal
Hind III
ORFs
2
CAT
3
4
5
1 (Δ 43 bp)
2 (Δ 373 bp)
3 (Δ 755 bp)
4 (Δ 914 bp)
5 (Δ 1261 bp)
6 (Δ 1615 bp)
7 (Δ 1940 bp)
8 (Δ 2164 bp)
9 (Δ 2838 bp)
Scale # of bp
43 373 755 914 1261 1615 1940 2164 2838
10
11
12
13
control reverse CAT
pCAT
pBS
IC
-0.5 0.0 0.5 1.0 1.5 2.0 2.5 3.0
CPM (X 10,000)
CAT Activity (3 replicates)

Fig. 2.5

Fig. 2.6 The effect of deletions from Sal I to Apa I on the levels of CAT expression from a polyhedron envelope promoter-CAT gene fusion. On the top left is shown a map of the parent construct. Below the parent map are schematic maps showing regions deleted from a set of deletion mutants. Below is shown a scale indicating the position and number of base pairs deleted for each mutation of the insert DNA. To the right of each map is shown a representation in a bar graph of the level of CAT expression from each clone. The number on the left in parenthesis is the deletion size in base pairs. Controls and details are the same as Fig. 2.5.

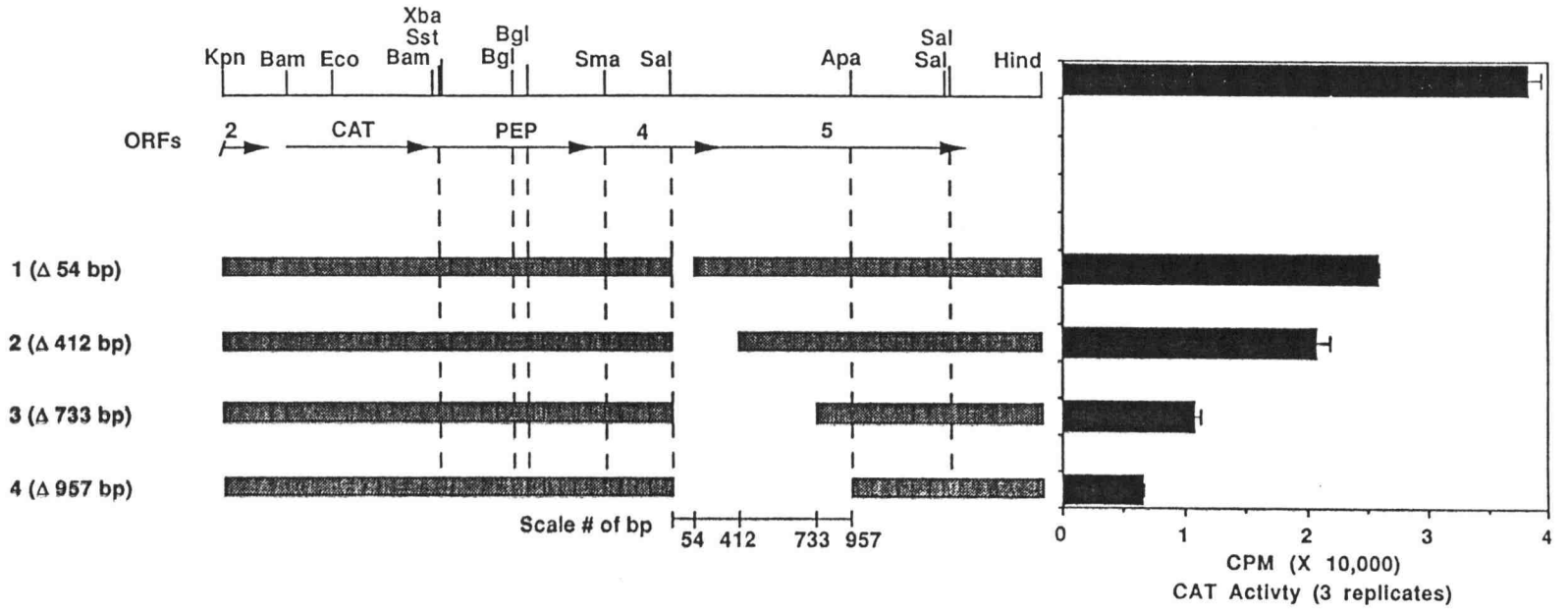

Kpn
Bam
Eco
Xba
Sst
Bam
Bgl
Bgl
Sma
Sal
Apa
Sal
Sal
Hind
ORFs
2
CAT
PEP
4
5
1 (Δ 54 bp)
2 (Δ 412 bp)
3 (Δ 733 bp)
4 (Δ 957 bp)
Scale # of bp
54
412
733
957
0
1
2
3
4
CPM (X 10,000)
CAT Activty (3 replicates)

Fig. 2.6

Fig 2.7 RNase protection analysis of the 3' deletion constructs. a) Schematic diagram of the RNase protection assay. On the top is a map showing the ORFs present in the parent construct. The region in which the deletions were made is indicated. The dashed lines shows the mRNA produced by the PEP-CAT promoter. The location of the complementary CAT RNA used in the RNase protection assay is shown. b) CAT mRNA RNase protection assay. Lanes 1 through 8 correspond to the deletion construct 1 through 8 in Fig 2.5. Lanes 1 through 4 (to the right) correspond to the deletion constructs 1 through 4 in Fig. 2.6. The standard curve and controls are not shown.

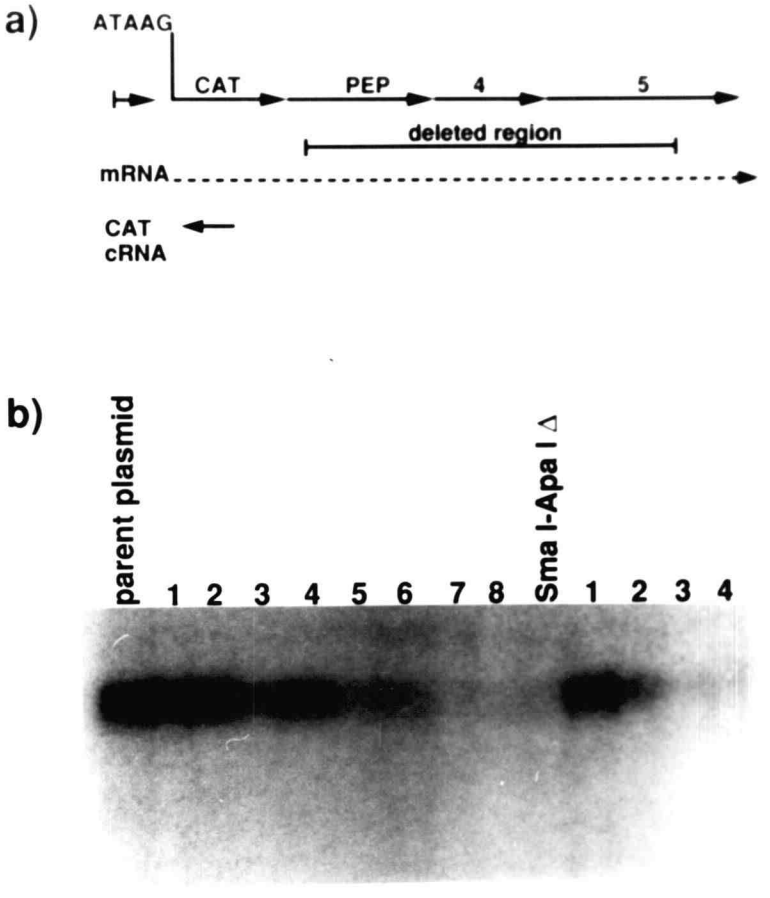

a)
ATAAG
CAT
PEP
4
5
deleted region
mRNA
CAT
cRNA
b)
parent plasmid
1 2 3 4 5 6 7 8
Sma I-Apa I Δ
1 2 3 4

Fig. 2.7

CHAPTER 3

A Baculovirus Capsid-Associated Protein: Immunocytochemical Characterization of p24

Christian H. Gross, Gregory M. Wolgamot, Rebecca L. Q. Russell and George F. Rohrmann

Department of Biochemistry and Biophysics
Oregon State University
Corvallis, Oregon 97331

Department of Agricultural Chemistry
Oregon State University
Corvallis, Oregon 97331

Abstract

An open reading frame (ORF 1) located upstream of the polyhedron envelope protein gene of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) was cloned in-frame into a *trp*E expression vector. The fusion protein produced by this construct was used for the preparation of a monospecific antiserum. Western blot analysis of OpMNPV infected *Lymantria dispar* cells and *Autographa californica* (AcMNPV) infected *Spodoptera frugiperda* cells detected a 24 kDa protein in extracts from cells late in infection. This antiserum also reacted with a 24 kDa protein in preparations of budded and polyhedra derived virus from OpMNPV and AcMNPV. Immunogold staining and electron microscopy indicated that the OpMNPV p24 is associated with the nucleocapsids of the budded and polyhedra derived viruses.

Introduction

The Baculoviridae are a family of insect pathogens with large double-stranded supercoiled DNA genomes of 88-163 kb. They are characterized by a complex infection cycle in which nucleocapsids are associated with two structurally distinct virion phenotypes. The polyhedra-derived virus phenotype (PDV) consists of nucleocapsids that become enveloped in the cell nucleus by a *de novo*-assembled membrane and then are occluded in polyhedron-shaped occlusion bodies or polyhedra. Polyhedra are released upon the death of the insect and thereby disseminate the virus between insects. The occluded virions remain viable for extended periods in the environment. In contrast, nucleocapsids associated with the budded virus (BV) phenotype are not occluded. After assembling in the nucleus, they are transported to the cell surface where they derive their membrane by budding through a virus-modified plasma membrane and disseminate the infection from cell to cell within the host insect.

Although the PDV and BV phenotypes serve different functions in the baculovirus infection cycle, the nucleocapsids appear to be structurally similar. In addition to containing the genome and a putative DNA binding protein of 6.5-7.5 kDa (Wilson et al., 1987; Russell and Rohrmann, 1990b; Maeda et al., 1991), PDV and BV nucleocapsids both appear to have at least two capsid-associated proteins. The p39 protein is a major capsid component and has been described from three NPVs (Blissard et al., 1989; Pearson et al., 1988; Thiem and Miller, 1989; Bjornson and Rohrmann, 1992b). In addition, another protein, p87, appears to be present in both AcMNPV and OpMNPV capsids (Müller et al., 1990; Lu and Carstens, 1991). In contrast, recent investigations by Kuzio et al. (1989), suggest that a protein called p74 may be a specific component of PDV nucleocapsids which is required for infectivity in insects but not cultured cells.

The protein p24 is encoded by the first open reading frame (ORF) in a series of five late-expressed ORF's of the polyhedron envelope protein gene region present in both the AcMNPV (Oellig et al., 1987,

Whitford et al., 1989) and OpMNPV (Gombart et al., 1989). In the E-strain of AcMNPV, the p24 gene is interrupted by a transposable element (Gombart et al., 1989a; Schetter et al., 1990) which has no evident effect on the ability of the virus to replicate under laboratory culture conditions. In addition, the gene has recently been sequenced in the *Lymantria dispar* multicapsid nuclear polyhedrosis virus (LdMNPV), and found to be lacking the amino terminal half of the open reading frame. In contrast to AcMNPV and OpMNPV where p24 is a late-expressed gene, in LdMNPV p24 is preceded by an RNA polymerase II promoter that is active in *in vitro* transcription systems, suggesting it is expressed as an early gene (Bjornson and Rohrmann, 1992b).

In this report we describe investigations on the possible role of the p24 gene in the infection cycle of the OpMNPV. This was accomplished by the production of a monospecific antiserum against a fusion protein that contained the complete p24 ORF. This antiserum was used for both western blot analysis and immunocytochemical localization of the protein using immunoelectron microscopy. These data indicate that p24 is a capsid-associated component of both phenotypes of AcMNPV and OpMNPV.

Materials and Methods

Virus and insect cells

The cloned isolate of OpMNPV was described in Quant-Russell et al. (1987). *Lymantria dispar* (Ld-652Y) cells were propagated in T-flasks using TNM-FH media (Summers and Smith, 1987) supplemented with 10% fetal bovine serum (FBS). For production of the budded virus, *Lymantria dispar* cells that had been adapted to shaker culture (a gift from Stephan Weiss, Gibco Laboratories) were grown in TNM-FH-10%FBS supplemented with 0.1% Pluronic-F68 (Gibco Laboratories). The AcMNPV E-2 strain was a gift from Dr L.Volkman. The AcMNPV E strain was a gift from Dr. W. Doerfler. *Spodoptera frugiperda* cells (Sf9 cells; ATTCC CRL 1711), obtained from Gibco/BRL, were grown in TNM-FH-10%FBS. All TNM-FH media was supplemented with penicillin G (50 units/ml), streptomycin (50 mg/ml, Whittaker Bioproducts), and fungizone (amphotericin B, 375 ng/ml, Flow Laboratories).

Purification of virus

For studies involving infected cells or budded virus, cells were infected with OpMNPV BV at a multiplicity of infection (moi) of 10 following the procedures of Bradford et al. (1990) unless otherwise indicated. For Western blot analysis, budded virus was isolated from two 150 ml shaker cultures of OpMNPV-infected *L. dispar* cells and AcMNPV-infected *Spodoptera frugiperda* cells at 72 hrs.p.i.. After low speed centrifugation to remove cell debris (2,000xg for 10 min), the supernatant was centrifuged at 80,000xg for 30 min. This pellet was resuspended in 3 ml of TE [10 mM Tris (pH 7.5), 1 mM EDTA] and further purified by centrifugation through a 38-52 % sucrose gradient at 80,000xg for 2 hrs. at 4 ^{o}C. The virion band was then removed, diluted in TE, and sedimented at 80,000xg for 1 hr to remove the sucrose. Viral occlusion bodies were isolated from infected *Orgyia pseudotsugata* larval cadavers as previously described (Rohrmann et al., 1978).

Construction of the *trp*E-p24 gene fusion

As part of another study (see Chapter 2), a plasmid (pBlueScribe - pBS, Stratagene) containing the ORF 1 gene region was modified by changing the ORF 1 late promoter element from GTAAG to GATCA creating a Bcl I site 17 nt upstream of the ORF 1 translational start site by site directed mutagenesis. This pBS plasmid was maintained in a *dam*$^-$ GM119 *E. coli* strain. A 683 base pair Bcl I-Pst I fragment containing the complete ORF1 gene was isolated and subcloned into the *trp*E expression vector pATH 11 (Koerner et. al, 1991) cut with Bam HI and Pst I (Fig. 3.1b). This resulted in a fusion protein of 58 kDa (37 kDa from *trp*E and 24 kDa from ORF 1) (Fig. 3.1c).

The *trp*E-p24 fusion protein: expression, isolation, and antibody production

To express the *trp*E-p24 fusion protein, cultures of *Escherichia coli* HB101 containing the *trp*E-p24 gene fusion plasmid were grown in modified M9 medium as described previously (Gross and Rohrmann, 1990). For isolation of fusion protein, bacteria from a 100 ml culture were harvested and then resuspended in 3 ml of 2 X SDS-PAGE buffer [0.125 M Tris (pH 7.5), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002% Bromophenol blue] and boiled for 5 min. A 1.5 ml sample was electrophoresed on a 20 cm long, 1.5 mm thick 10% SDS-polyacrylamide gel in a Bio-Rad Protean II electrophoresis unit at 35 volts for 12-15 hrs. The fusion protein band was identified by cutting three thin strips from the gel (one on each margin and one in the center) and staining with Coomassie brilliant blue. The location of the *trp*E-p24 fusion was determined and the band was excised from the unstained gel and electroeluted overnight using a Schleicher and Schuell Elutrap apparatus at 100 V. A New Zealand White female rabbit was initially injected with approximately 500 µg [as determined by Bradford protein assay (BioRad)] of fusion protein emulsified in complete Freund's adjuvant. Subsequent injections of approximately 200 µg of fusion protein emulsified in incomplete Freund's adjuvant

were given at biweekly intervals starting the third week after initial injection. Immune serum was collected 7 days after the third boost. Preimmune serum was collected before the initial injection.

Western blots

For analysis of the expression of the p24 protein in infected cells, *Lymantria dispar* cells infected at moi 10 were collected at different times post infection, lysed in 2X SDS-PAGE sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue) and an amount of protein equivalent to approximately 4.8 X 10^4 cells per lane was electrophoresed on a 10% SDS-polyacrylamide gel (Laemmli, 1970). Western blots were prepared as previously described (Quant-Russell et al., 1987). The *trp*E-p24 antiserum was diluted 1:1000 in TBS-T (20 mM Tris (pH 7.5), 500 mM NaCl, 0.05% Tween 20) and incubated on Western blots for 2 hours. Blots were rinsed in three 10 mins washes of TBS-T and incubated 1-2 hours in a 1:7500 dilution of goat anti-mouse IgG conjugated with alkaline phosphatase (Promega). Blots were rinsed three times for 10 min in TBS-T. Immunoreactive proteins were detected by incubating the blot in a substrate solution of 330 mg/ml nitro blue tetrazolium and 165 mg/ml 5-bromo-4-chloro-3 indolyl phosphate.

Immunoelectron microscopy

Lymantria dispar cells were infected with OpMNPV at an m.o.i. of 100. The cells were harvested at 84 hrs.p.i., fixed in 2.5% glutaraldehyde, dehydrated with ethanol, and embedded in LR White resin (Russell and Rohrmann, 1990a). The *trp*E-p24 antiserum was used at a dilution of 1:1000. Immunogold labeling was carried out as previously described using 10 nm gold particles (Russell and Rohrmann, 1990a).

Results and Discussion

Characterization of p24 expression

To investigate the expression of p24, an antiserum was generated against the *trp*E-p24 fusion protein produced in a pATH 11 bacterial expression plasmid (Fig. 3.1). The time course of expression was initially examined using Western blot analysis of OpMNPV or AcMNPV infected cell extracts from different times post infection. In both OpMNPV and AcMNPV (E-2 strain) infected cells, p24 was first detected at 24 hrs.p.i. and accumulated through 120 hrs.p.i., the last timepoint assayed (Fig. 3.2a,b). These data are consistent with p24 being expressed as a late gene in these viruses. To determine if p24 is associated with a structural component of virions, preparations of BV and occluded virus (OV) from OpMNPV and AcMNPV were subjected to Western blot analysis. This data indicates that p24 is present in purified BV (Fig. 3.2c lane 1,3) and OV (lane 2,4) preparations from OpMNPV and AcMNPV. Although the ORF 1 gene product shows an apparent molecular weight of 24 kDa on Western blots, the molecular weight predicted from the sequence is 21.2 kDa. To determine whether p24 was N-glycosylated, budded virus was digested with N-glycanase (Genzyme Corp.) according to the manufacturer's instructions. After digestion, treated and untreated BV preparations were electrophoresed on SDS-PAGE gels and Western blot analysis was performed using the p24 antiserum and an antiserum to gp64 to detect a glycosylated control (gp64) in the same samples. Although the gp64 protein was reduced in size to about 55 kDa by digestion with N-glycanase, p24 was unaffected indicating that it is not N-glycosylated (data not shown). Similarly, no evidence of N-glycosylation was found when virus was grown in the presence of tunicamycin (data not shown).

Immunoelectron microscopy of p24

Localization of p24 in OpMNPV infected *L. dispar* cells was examined by immunogold staining and electron microscopy. The p24 antiserum stained chromatin-like material around the margins of the

nuclei of cells at 84 hrs. p.i. (Fig. 3.3a). In addition, it showed specific staining of both non occluded and occluded nucleocapsids present in the nucleus (Fig. 3.3a to d) and also nucleocapsids present in the cytoplasm (Fig. 3.3d). No staining of cytoplasmic structures other than virions was evident (Fig. 3.3d). The staining is randomly distributed over the surface of the nucleocapsids suggesting p24 is a component of the capsid rather than a specialized end structure. Neither uninfected or infected cells showed significant staining with the *trp*E-p24 or anti-*trp*E antisera, respectively (data not shown).

In this investigation of the localization of the p24 protein, we have demonstrated that p24 is specifically associated with nucleocapsids of both BV and PDV in AcMNPV and OpMNPV. These data suggest that baculovirus capsids are composed of at least three components; p39 (Blissard et al., 1989; Pearson et al., 1988; Thiem and Miller, 1989), p87 (Müller et al., 1990; Lu and Carstens, 1991), and p24. Although p24 was shown in this study to be highly specific to baculovirus nucleocapsids, p24 is interrupted by a transposable element in the E strain of AcMNPV (Gombart et al., 1989a; Oellig et al., 1987; Schetter et al., 1990). Using *trp*E-SLP antiserum and Western blot analysis the expression of the interrupted p24-ORF was not detected (data not shown). In the CI 5-6 strain of LdMNPV the amino terminal half of the p24 homologue is not present (Bjornson and Rohrmann, 1992b). These data suggest that a complete p24 ORF is not essential for viability of these two virus strains. In addition, understanding the possible role of the p24 protein is complicated by the observation that the LdMNPV p24 gene appears to be regulated by an RNA polymerase II (early) promoter element (Bjornson and Rohrmann, 1992b) rather than being expressed as a late gene as observed in OpMNPV (Gombart et al, 1989a). The implications of the variablilty of the structure and expression of this gene remains to be elucidated.

Fig. 3.1 Construction of a *trp*E-p24 gene fusion. a) Restriction map of the p24 (ORF 1) region. b) Schematic diagram of the fusion of the *trp*E protein to ORF 1 in the pATH 11 expression vector. c) Nucleotide sequence at the fusion junction.

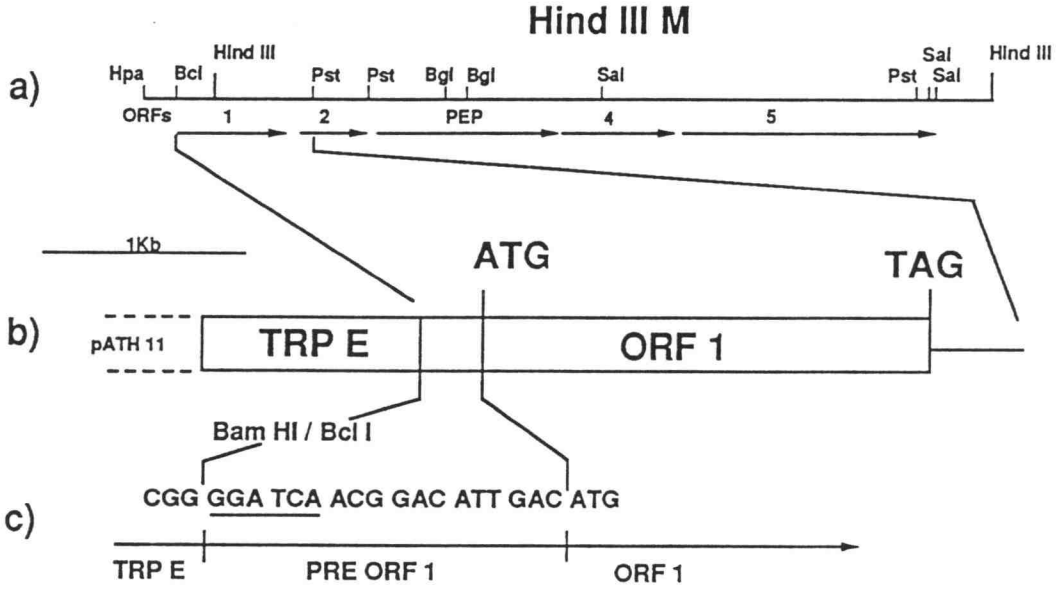

Hind III M
a)
Hpa
Bcl
Hind III
Pst
Pst
Bgl
Bgl
Sal
Pst
Sal
Sal
Hind III
ORFs
1
2
PEP
4
5
1Kb
ATG
TAG
b)
pATH 11
TRP E
ORF 1
Bam HI / Bcl I
c)
CGG GGA TCA ACG GAC ATT GAC ATG
TRP E
PRE ORF 1
ORF 1

Fig. 3.1

Fig. 3.2 Western blot analysis of p24 in OpMNPV infected *Lymantria dispar* cells, AcMNPV infected *Spodoptera frugiperda* cells and viral phenotypes. a) Time course of p24 expression in OpMNPV infected cells. b) Time course of p24 expression in AcMNPV infected cells. The number on the top of each well indicates hours post infection when proteins were isolated. Proteins from uninfected cells (U) are shown in first lane. c) Western blot analysis of the p24 in OpMNPV or AcMNPV budded and occluded viral phenotypes. Lanes: 1,3 Purified BV from OpMNPV (OpBV), and AcMNPV (AcBV) respectively; 2,4 Polyhedra from OpMNPV (OpOV) and AcMNPV (AcOV) respectively. Polyhedra were solubilized in 2X sample buffer and boiled for 5 mins. The *trp*E-p24 antiserum was used at a 1:1000 dilution. For all blots, sizes (kDa) of the protein standards are indicated on the left and the location of the p24 is indicated on the right.

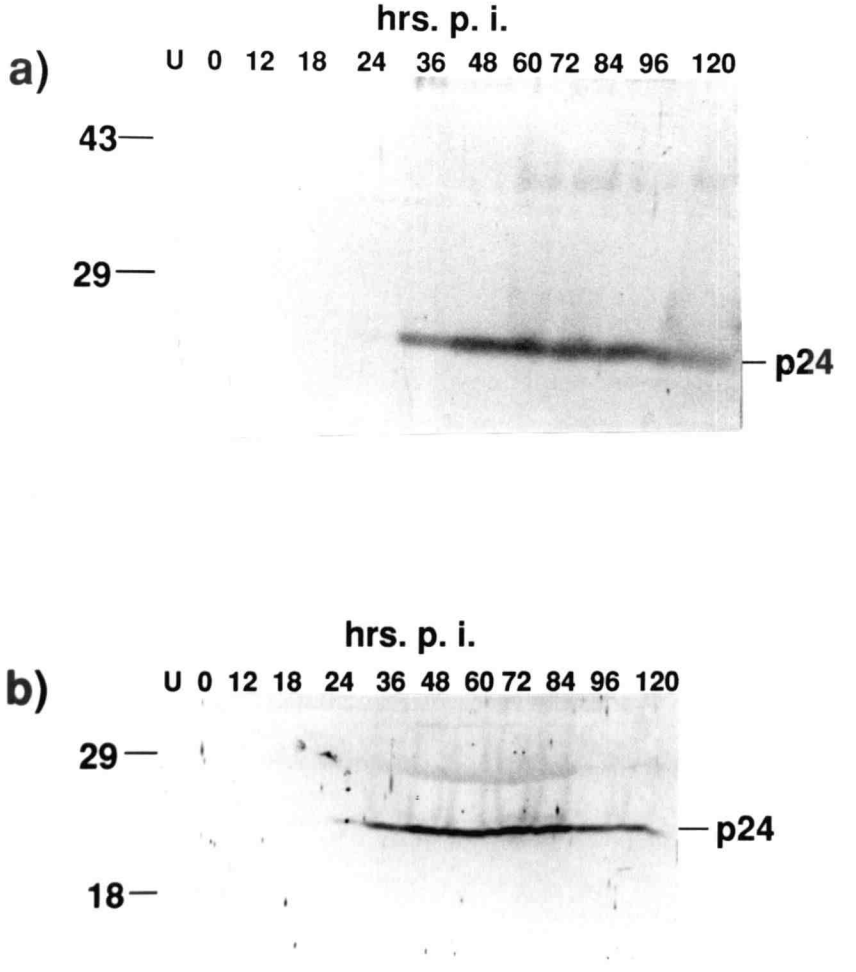

hrs. p. i.
a)
U 0 12 18 24 36 48 60 72 84 96 120
43
29
p24
hrs. p. i.
b)
U 0 12 18 24 36 48 60 72 84 96 120
29
18
p24

Fig. 3.2

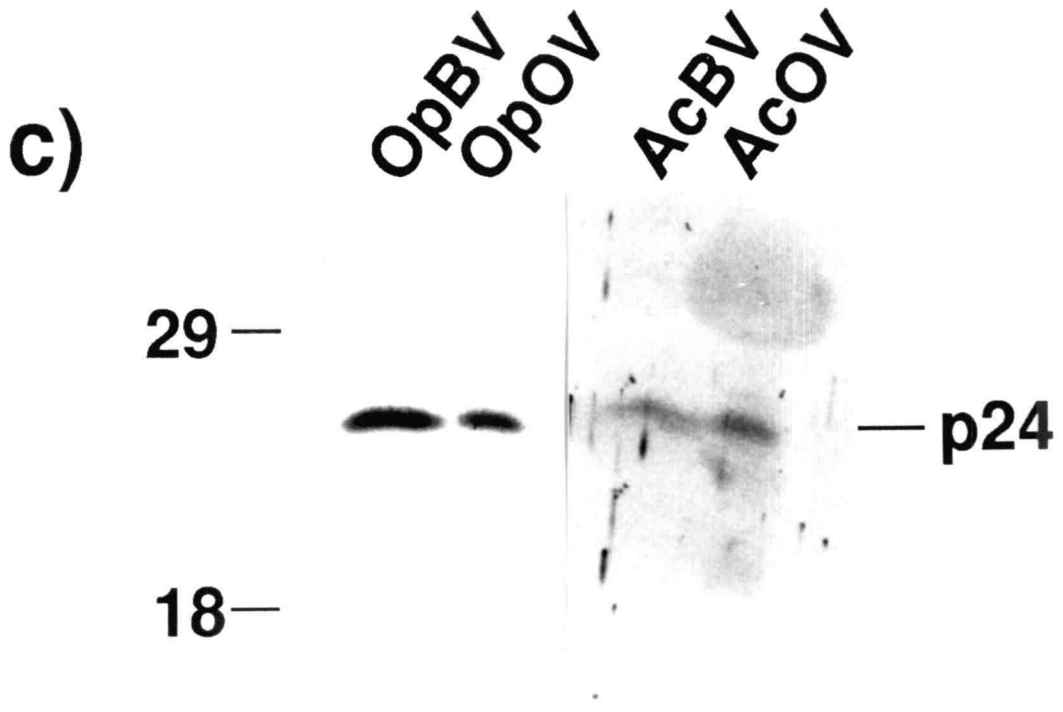
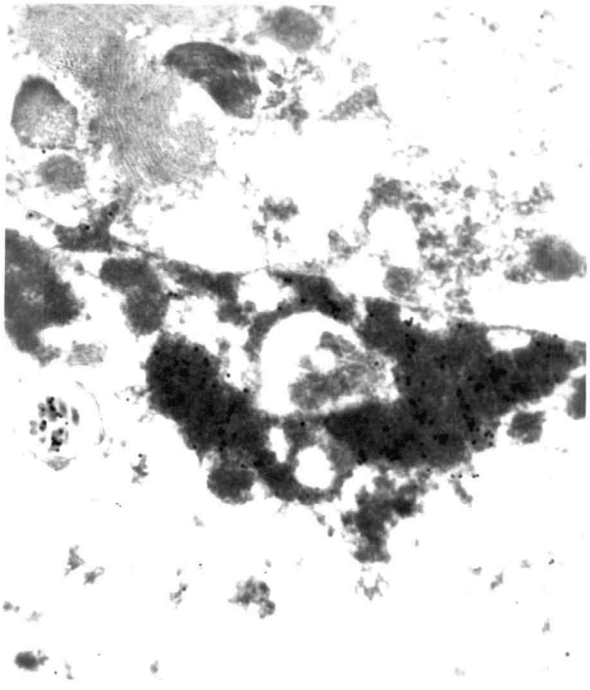


Fig. 3.2 continued

Fig. 3.3 Immunogold staining of OpMNPV infected *Lymantria dispar* cells. a) nucleocapsids and chromatin-like material, b and c) occlusion bodies, d) occlusion body in the nucleus and budded virus in the cytoplasm. The p24 antiserum was diluted 1:2000 and the second antibody was diluted 1: 20. All samples are from 84 hrs.p.i.. The scale: 1 cm is equal to a) 0.3 μm, b) 0.2 μm, c) 0.3 μm, d) 0.2 μm.

a)



b)

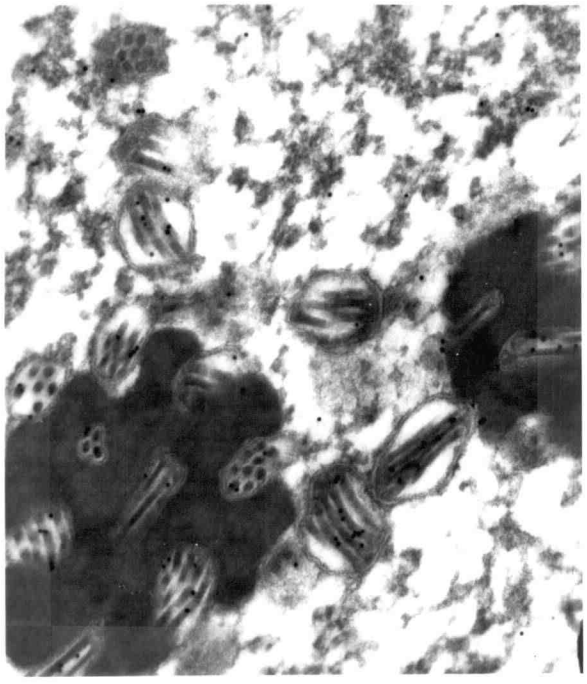


Fig. 3.3

c)

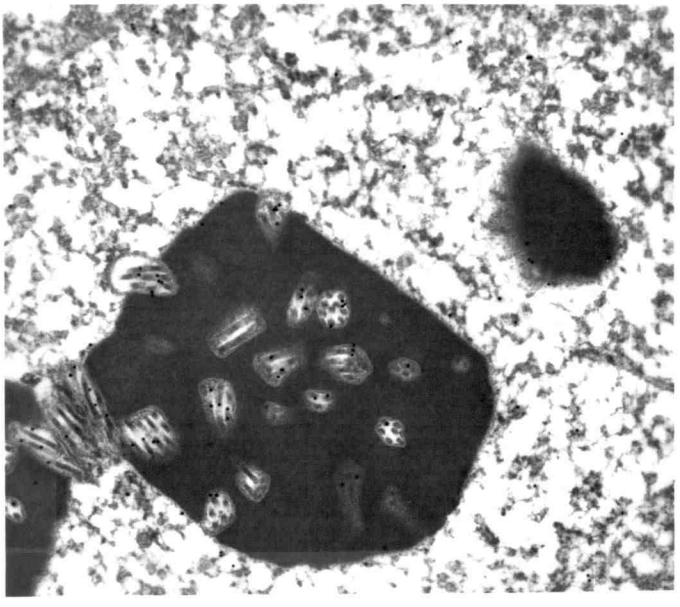


Fig. 3.3 continued

d)

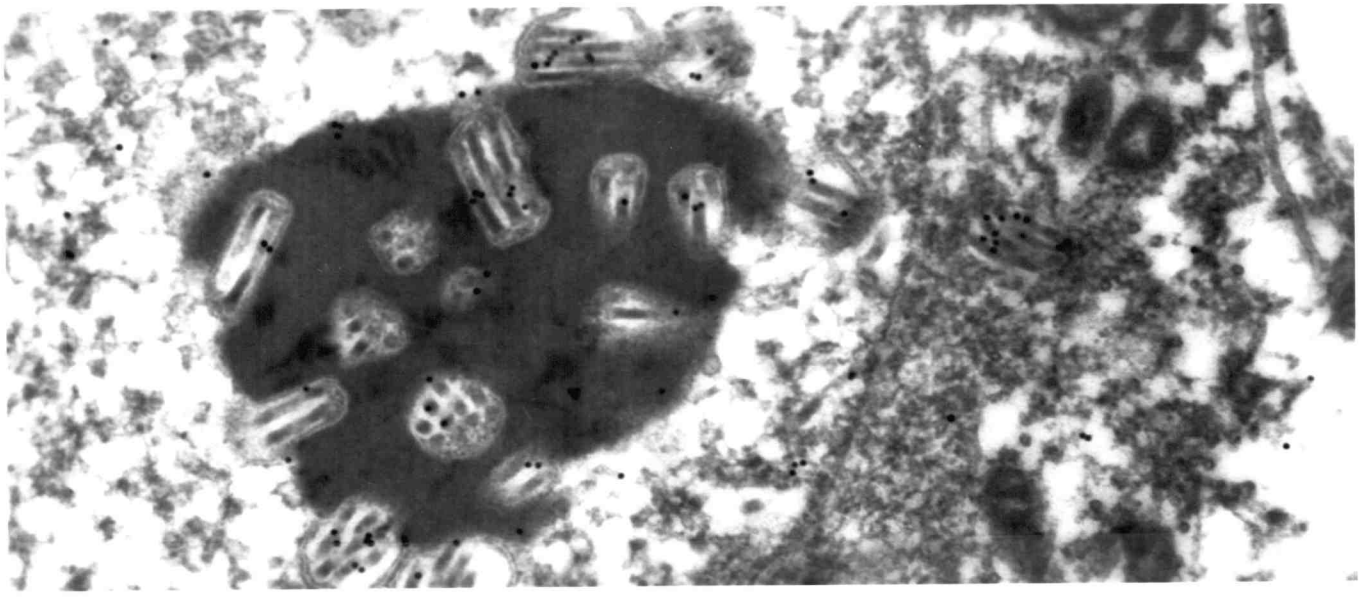


Fig. 3.3 continued

CHAPTER 4

A Baculovirus 16 kDa Glycoprotein Localizes to the Nuclear Membrane of Infected Cells

Christian H. Gross, Gregory M. Wolgamot, Rebecca L. Q. Russell, Margot N. Pearson and George F. Rohrmann

Department of Biochemistry and Biophysics
Oregon State University
Corvallis, Oregon 97331

Department of Agricultural Chemistry
Oregon State University
Corvallis, Oregon 97331

Abstract

An open reading frame (ORF 2) located upstream of the polyhedron envelope protein gene region of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) was cloned in-frame into a *trp*E gene fusion vector. The fusion protein produced by this construct was used for the production of a monospecific antiserum. Western blot analysis of OpMNPV infected *Lymantria dispar* cells detected a 16 kDa protein at 24 hours post infection. The 16 kDa protein was determined to have N-glycosylation. Immunofluorescence microscopy localized the 16 kDa protein to foci of intense cytoplasmic staining near the nuclear membrane. Immunogold staining and electron microscopy indicated that the 16 kDa protein was associated with the nuclear membrane, with lamellar-like membranous structures peripheral to the nuclear membrane, and with the envelopes of the budded virus in transit through the cytoplasm. The 16 kDa protein was not associated with the purified budded or polyhedra derived virus virions.

Introduction

Nuclear polyhedrosis viruses (NPVs) are members of the *Baculoviridae*, a family of large double-stranded DNA viruses with a genome sizes of 88-160 kbp. They are pathogenic for arthropods, primarily insects of the orders Lepidoptera, Hymenoptera, and Diptera. NPVs replicate in the host cell nucleus and are characterized by the occlusion of virions in polyhedron-shaped structures that are composed predominantly of a single protein, polyhedrin, of about 29 kDa. The infection cycle of the *Autographa californica* multicapsid NPV (AcMNPV) is complex and involves the production of two structurally distinct virion phenotypes. The polyhedra-derived virus phenotype (PDV) is the occluded form of the virus and has an envelope that is assembled *de novo* in the cell nucleus. Upon ingestion of polyhedra, the PDV are released when the polyhedra are dissolved by the high pH of the insect midgut. The PDV invade and undergo an initial round of replication in midgut cells and then spread the infection to other cells throughout the insect. The virions that spread the infection are not occluded and are called budded virus (BV). Nucleocapsids destined to become budded virus, bud through the nuclear membrane and temporarily acquire both the inner and outer nuclear envelope. This double-membrane is then apparently lost as the nucleocapsids are transported through the cytoplasm to the plasma membrane (Granados and Lawler, 1981; Kawamoto et al., 1977b). The nucleocapsids again acquire an envelope when they bud through the cytoplasmic membrane that has been modified by a viral glycoprotein, gp64. Although gp64 has been demonstrated to play an important role in the life cycle, the role of other glycoproteins in the NPV life cycle has not been well-documented. Recently Roberts (1989) reported that a 12 kDa protein (open reading frame (ORF) 2) is glycosylated in AcMNPV to a MW of 16 kDa. ORF 2 is located in a series of 5 ORFs that are oriented in the same direction at about map unit 81 in the AcMNPV genome (Oellig et al, 1987). This region has also been sequenced in the genome of the NPV of *Orgyia pseudotsugata* (OpMNPV) and ORF 2 shows 72% amino acid sequence identity between the AcMNPV and OpMNPV making it one of the most highly conserved baculovirus

ORFs (Gombart et al, 1989b). Roberts (1989) investigated gp16 in AcMNPV infected *Spodoptera frugiperda* cells by immunofluorescence microscopy, and found gp16 to be primarily localized to the cytoplasmic perinuclear and nuclear membrane regions. In order to further characterize the role of glycoproteins in the NPV infection cycle, we have produced a monospecific antiserum to a *trp*E-ORF 2 fusion protein. The antiserum was used to investigate the expression and localization of the ORF 2 protein (gp16) in OpMNPV infected *Lymantria dispar* cells.

Materials and Methods

Cells and virus

The cloned isolate of OpMNPV was described in Quant-Russell et al (1987). *Lymantria dispar* (Ld-652Y) cells were propagated in T-flasks and using TNM-FH media (Summers and Smith, 1987) supplemented with 10% fetal bovine serum (FBS). For production of budded virus, *Lymantria dispar* cells that had been adapted to shaker culture (a gift from Stephan Weiss, Gibco laboratories) were grown in TNM-FH-10% FBS supplemented with 0.1% Pluronic-F68 (Gibco Laboratories).

Purification of virus

For studies involving infected cells or budded virus, cells were infected with OpMNPV BV at a multiplicity of infection (moi) of 10 following the procedures of Bradford et al. (1990), unless otherwise indicated. For Western blot analysis, BV was isolated from two 150 ml shaker cultures of OpMNPV-infected *L. dispar* cells at 72 hrs.p.i.. After low speed centrifugation to remove the cells (2000xg for 10 min), the supernatant was centrifuged at 80,000xg for 30 min. This preparation was resuspended in 3 ml of TE (10 mM Tris [pH 7.5], 1 mM EDTA). Samples of this preparation were further purified by centrifugation through a 38-52% sucrose gradient at 80,000xg for 2 hr at 4 ^{o}C, and the virion band was removed, diluted in TE (10 mM Tris pH 7.5, 1 mM EDTA), and sedimented at 80,000xg for 1 hr to remove sucrose. Viral occlusion bodies were isolated from infected *Orgyia pseudotsugata* larval cadavers as previously described (Rohrmann et al., 1978).

Construction of the *trp*E-gp16 gene fusion

For another study (see Chapter 2), a plasmid (pBlueScribe - pBS, Stratagene) containing the ORF 2 gene region was modified at two positions by site directed mutagenesis. The ORF 2 late promoter was changed from ATAAG to AGATC, which created at Bgl II site 18 nt

upstream of the ORF 2 translational start site and a Bam HI site was positioned at the ORF 3 (polyhedron envelope protein) translational start site (the sequence ATGACGCC was changed to ATGGATCC) (Fig. 4.1a). A 375 base pair Bgl II-Bam HI fragment was isolated and subcloned into pBS cut with Bam HI. The Eco R1-Hind III insert from a subclone with the 5' end of ORF 2 adjacent to the EcoRI site in the polylinker was subcloned into the *trp*E expression vector pATH 3 (Koerner et al., 1991) (Fig. 4.1b). The result was *trp*E gene fusion containing the complete ORF 2 gene (Fig. 4.1c).

The *trp*E-gp16 fusion protein: expression, isolation, and antibody production

To express the *trp* E-gp16 fusion protein, cultures of *Escherichia coli* HB101 carrying the plasmid containing the *trp*E-gp16 gene fusion were grown in modified M9 medium as described previously (Gross and Rohrmann, 1990). For isolation of fusion protein, bacteria from a 100 ml culture were resuspended in 3 ml of 2 X SDS-PAGE buffer (0.125 M Tris (pH 7.5), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002% Bromophenol blue) and boiled for 5 min. A 1.5 ml sample was electrophoresed on a 20 cm long, 1.5 mm thick 10% SDS-polyacrylamide gel in a Protean II (Bio-Rad) electrophoresis unit at 35 volts for 12-15 hr. The location of the fusion protein band was identified by staining a portion of the gel with Coomassie brilliant blue, the band was excised, and electroeluted at 100 V overnight by using a Schleicher and Schuell Elutrap apparatus. A New Zealand White female rabbit was initially injected with approximately 500 µg (as determined by a Bradford protein assay [BioRad]) of fusion protein emulsified in complete Freund's adjuvant followed by 200 µg emulsified in incomplete Freund's adjuvant at biweekly intervals starting the third week after initial injection. Immune serum was collected 7 days after the third boost. Preimmune serum was collected before the initial injection.

Western Blots

For analysis of the expression of the gp16 protein, *Lymantria dispar* cells from different times post infection (m.o.i. 10) were lysed in 2X SDS-PAGE sample buffer and an amount of protein equivalent to approximately 4.8 X 10^4 cells was electrophoresed on each lane of a 10 % SDS-polyacrylamide gel (Laemmli, 1970). Western blots were prepared as previously described (Quant-Russell et al., 1987). The *trp* E-ORF 2 antiserum was diluted 1:1000 in TBS-T (20 mM Tris [pH 7.5], 500 mM NaCl, 0.05% Tween 20) and incubated on Western blots for 2 hours. Blots were rinsed in three 10 min washes of TBS-T and incubated 1-2 hours in a 1:7500 dilution of goat anti-mouse IgG conjugated with alkaline phosphatase (Promega). Immunoreactive proteins were detected by incubating the blot in a substrate solution of 330 µg/ml nitro blue tetrazolium and 165 µg/ml 5-bromo-4-chloro-3 indolyl phosphate.

N-linked glycosylation assay

For the N-glycosylation studies, *Lymantria dispar* cells were infected at an m.o.i. of 10 and subsequently grown in TNM-FH-10% FBS medium containing 10 µg of tunicamycin per ml prepared from a 1mg/ml stock in dimethyl sulfoxide. At 60 hrs.p.i. cells were collected and resuspended in electrophoresis sample buffer, separated by SDS-PAGE and Western blot analysis was performed.

Immunofluorescence microscopy

Immunofluorescence microscopy studies of OpMNPV-infected cells were performed as previously described (Pearson et al., 1988). Cells were infected at an m.o.i. of 100 and harvested at 48 and 76 hrs.p.i.. The *trp*E-gp16 antiserum was used at a dilution of 1:1000 in buffer A.

Immunoelectron microscopy

Lymantria dispar cells were infected with OpMNPV at an m.o.i. of 100. Immunogold labeling was carried out as previously described using 10 nm gold particles (Russell and Rohrmann, 1990a). The cells were harvested and fixed in 2.5% glutaraldehyde, dehydrated with ethanol, and embedded in LR White resin. The *trp*E-gp16 antiserum was used at a dilution of 1:1000.

Results and Discussion

Characterization of gp16 expression and glycosylation

To study the expression of gp16, a specific antiserum was generated against the *trp*E-gp16 fusion protein produced in a pATH 3 bacterial expression plasmid (Fig. 4.1). The expression of gp16 in OpMNPV infected *Lymantria dispar* cells was investigated by reacting Western blots of cell extracts from various times post infection with *trp*E-gp16 antiserum. The gp16 was first detected at 24 hrs.p.i. and increased through 120 hrs.p.i., the last time point assayed (Fig. 4.2a). This data was consistent with gp16 being expressed as a late gene. The transcriptional start site of gp16 has previously been mapped to the late promoter sequence ATAAG (Gombart et al., 1989). To determine if gp16 was associated with the structural components of virions, preparations of BV and occluded (OV) from OpMNPV were subjected to Western blot analysis. These data indicated that gp16 was not present in purified BV and OV preparations (Fig. 4.2b lanes 1,2) but was present in the infected cell control (lane 3).

The ORF 2 gene product has been shown to be N-glycosylated in AcMNPV (Roberts, 1989). Broad ORF 2 bands were detected on Western blots of OpMNPV infected cell extracts indicating that the protein may be heterogeneous in size. To determine whether OpMNPV gp16 was N-glycosylated, cells were infected in the presence of tunicamycin. At 60 hrs.p.i. extracts from cells treated with and without tunicamycin were electrophoresed on SDS-PAGE gels and Western blot analysis was performed. The untreated gp16 protein (-Tun Fig. 4.2c) showed a slower mobility than the tunicamycin treated gp16 protein (+Tun, Fig. 4.2c) indicating that gp16 is N-glycosylated. From the predicted sequence (Gombart et al., 1989b) OpMNPV gp16 has one N-glycosylation site this site is conserved in AcMNPV gp16.

Immunofluorescence microscopy of gp16

To trace expression and location of the gp16 in infected *L. dispar* cells, cells were examined by immunofluorescence microscopy using the *trp*E-gp16 antiserum. At 48 and 76 hrs.p.i., the gp16 was concentrated in foci of intense nuclear membrane staining and some cytoplasmic staining (Fig. 4.3a, b). Only minimal staining of unifected cells was observed (Fig. 4.3c). These immunofluorescence results agreed with those reported in AcMNPV (Roberts, 1989) in which gp16 was primarily localized in cytoplasmic perinuclear and nuclear membrane regions. Anti-*trp*E (an antiserum produced against the non-fused truncated *trp*E protein) and preimmune serum controls showed no staining of infected cells (data not shown).

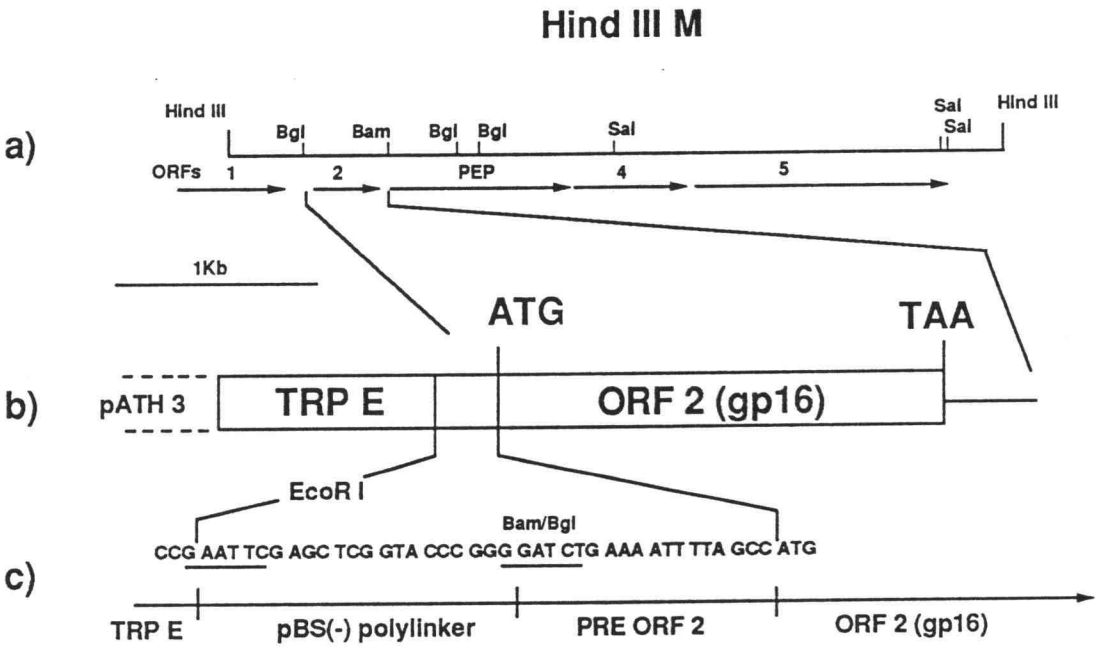
Immunoelectron microscopy of gp16

To more precisely localize gp16 in OpMNPV infected *Lymantria dispar* cells, cells were stained with immunogold and examined by electron microscopy. At 48 hrs.p.i., gp16 was found to be associated with aggregates of membrane-like structures located in the cytoplasm near the nuclear membrane (Fig. 4.4a). It is unclear whether these structures are generated by the viral infection or are a modification of an existing cellular structure (e.g. endoplasmic reticulum), although such structures are not observed in uninfected cells (data not shown). At 72 hrs.p.i., gp16 was distributed along the nuclear membrane (Fig. 4.4b) and nucleocapsids appeared to be budding through the nuclear membrane where it was associated with gp16 (Fig. 4.4b, d to e). Nucleocapsids within envelopes containing gp16 were also observed in the cytoplasm (Fig. 4.4c to e) at 72 and 84 hrs.p.i. These data indicate that gp16 may be involved in the movement of nucleocapsids through the nuclear membrane. Gp16 is also a component of the virions present in the cytoplasm suggesting that gp16 may be involved in the transit of virions to the cell surface. The absence of gp16 in virions that have budded from the plasma membrane suggests that the gp16-associated envelope observed at the nuclear membrane was lost during the morphogenesis of the BV envelope. Neither uninfected or

infected cells showed significant staining with the *trp*E-p24 or anti-*trp*E antisera, respectively (data not shown).

In this investigation of the localization of the gp16 protein, we have shown that gp16 was associated with cytoplasmic membrane-like structures found in association with the nuclear membrane. gp16 was also associated with the envelope of nucleocapsids after they budded through the nuclear membrane. These results support previous reports on NPV infection cycles, which have shown that nucleocapsids destined to become budded virus bud through the nuclear membrane and temporarily acquire both the inner and outer nuclear envelope (Granados and Lawler, 1981; Kawamoto et al., 1977b). This double-membrane is then apparently lost as the nucleocapsids are transported through the cytoplasm to the plasma membrane. We have examined purified preparations of BV by Western blot analysis and could not detect gp16. This would be expected if the envelope is lost as the nucleocapsid is transported to the cytoplasmic membrane.

Fig. 4.1 Construction of a *trp*E-gp16 gene fusion. a) Restriction map of the gp16 (ORF 2) region. b) The Bgl II-Bam HI fragment encoding the gp16 ORF was initially cloned into pBS to obtain an EcoRI site in the proper reading frame, then removed by digestion with EcoR I and Hind III and cloned into the pATH 3 expression vector. This resulted in a fusion protein of 49 kDa (37 kDa from trpE and 12 kDa from ORF 2). c) Nucleotide sequence at the fusion junction.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a)
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Bgl
Bam
Bgl
Bgl
Sal
Sal
Sal
Hind III
ORFs
1
2
PEP
4
5
1Kb
ATG
TAA
b)
pATH 3
TRP E
ORF 2 (gp16)
EcoR I
Bam/Bgl
c)
CCG AAT TCG AGC TCG GTA CCC GGG GAT CTG AAA ATT TTA GCC ATG
TRP E
pBS(-) polylinker
PRE ORF 2
ORF 2 (gp16)

Fig. 4.1

Fig. 4.2 Western blots of a time course of infection and virion phenotype of gp16. a) Western blot showing gp16 expression in OpMNPV infected *Lymantria dispar* cells. The numbers at the top of the wells indicates hours post infection. Proteins from uninfected cells (U) are shown in the first lane. b) Western blot showing gp16 was not present in the purified preparation of BV and OV (lane 1 and 2) but was present in the 60 hrs.p.i. infected cell control (IC, lane 3). The p24 protein was a positive control and present in both BV and OV (lane 1 and 2). c) Analysis of N-linked glycosylation of gp16 in OpMNPV infected *Lymantria dispar* cells (see methods). The figure shows a Western blot analysis of the gp16 treated without tunicamycin (-Tun) and with tunicamycin (+Tun). Sizes (kDa) of the protein standards are indicated on the left and the location of gp16 is indicated on the right. The *trp*E-gp16 antiserum was diluted 1:1000.

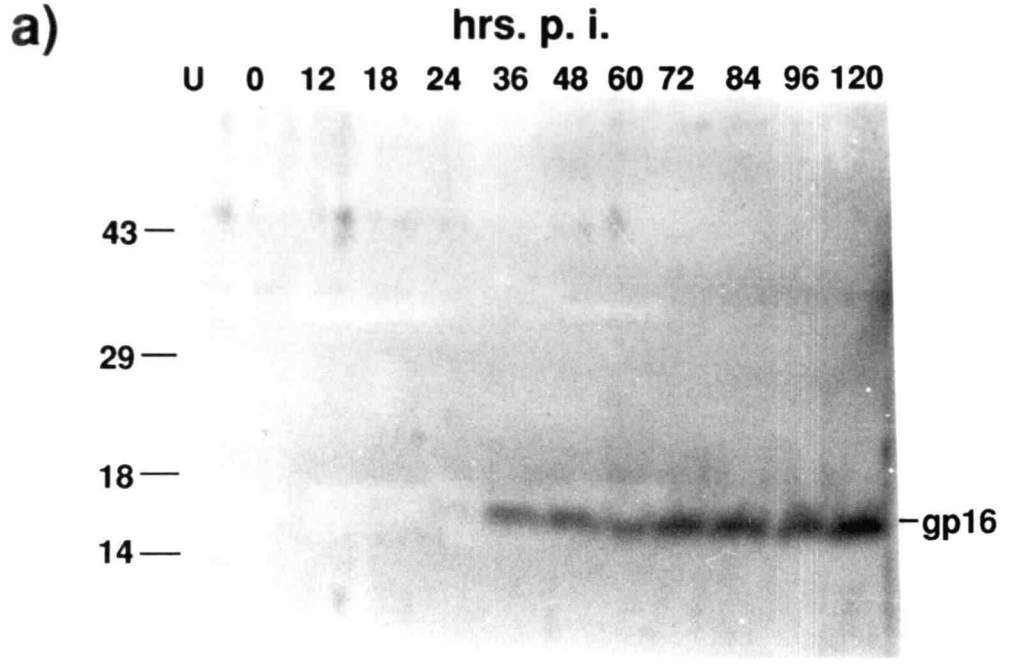


Fig. 4.2

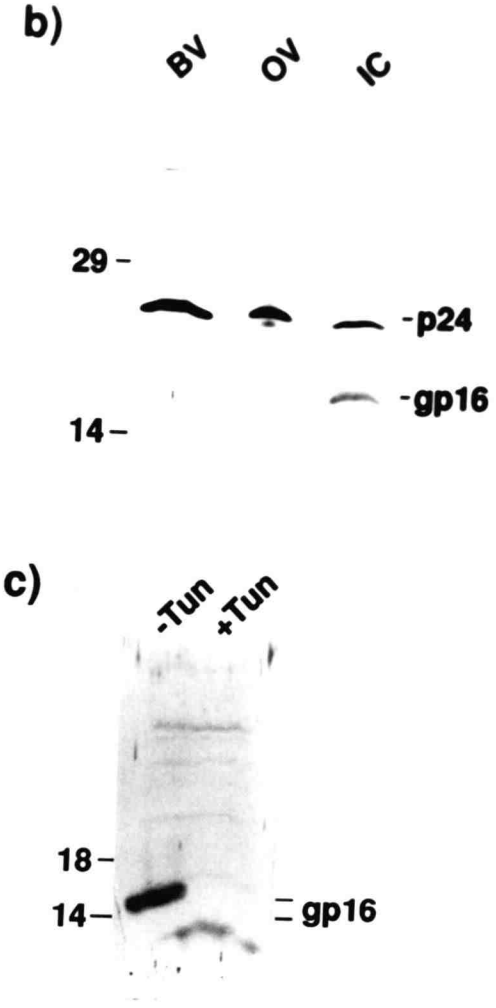
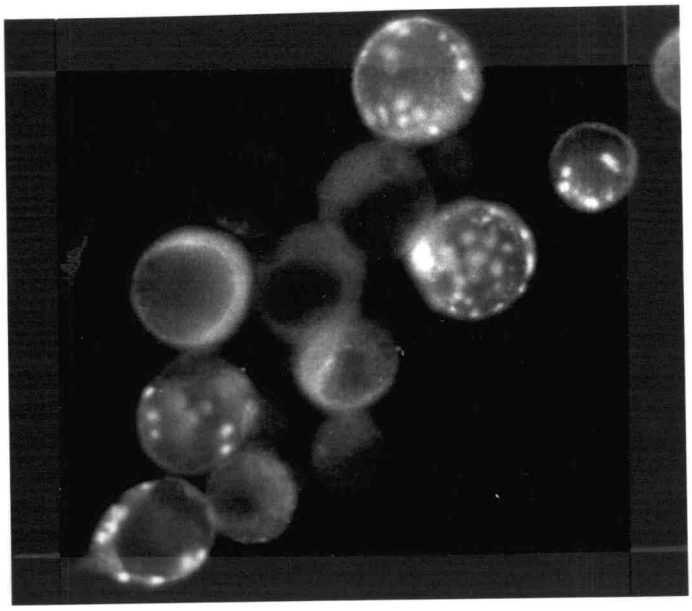


Fig. 4.2 continued

Fig. 4.3 Indirect immunofluorescence staining of OpMNPV infected *Lymantria dispar* cells at various times p.i.. a) 48 hrs.p.i. b) 76 hrs.p.i. c) Uninfected cells.

a)



b)

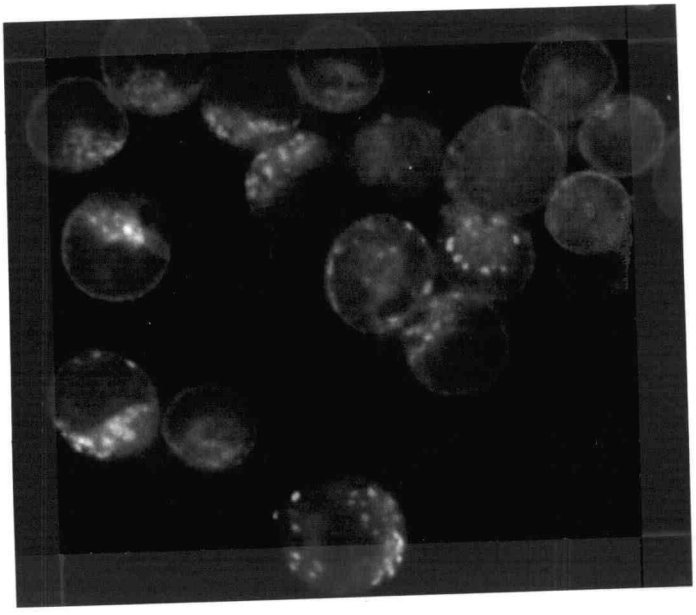


Fig. 4.3

c)

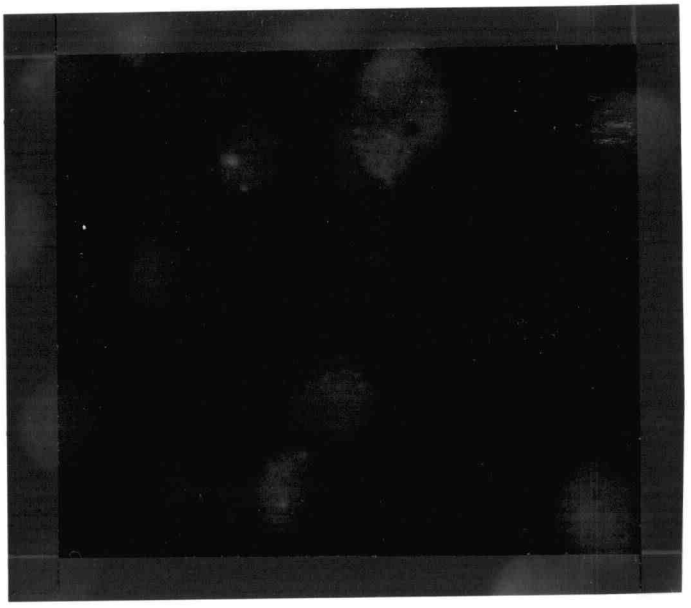
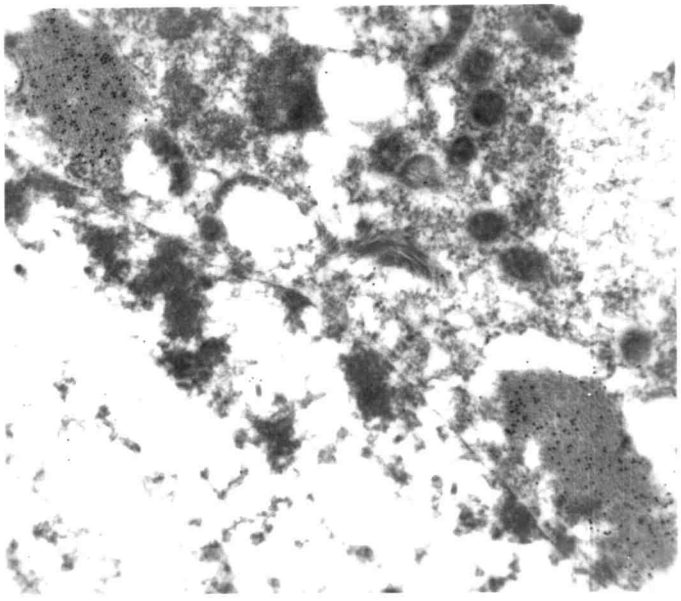


Fig. 4.3 continued

Fig. 4.4 Immunogold staining of OpMNPV infected *Lymantria dispar* cells at various times p.i.. a) gp16 associated with membrane-like structures near nuclear membrane (48 hrs.p.i.). b-d) gp16 associated with membrane-like structure and the nuclear membrane (72 hrs.p.i.). e) gp16 associated with membrane-like structure, nuclear membrane and enveloped nucleocapsids in cytoplasm (84 hrs.p.i.). The *trp*E-gp16 was diluted 1:2000 and the second antibody was diluted 1:20. The scale: 1 cm is equal to a) 0.4 μm b) 0.1 μm c) 0.2 μm d) 0.1 μm e) 0.1 μm.

a)



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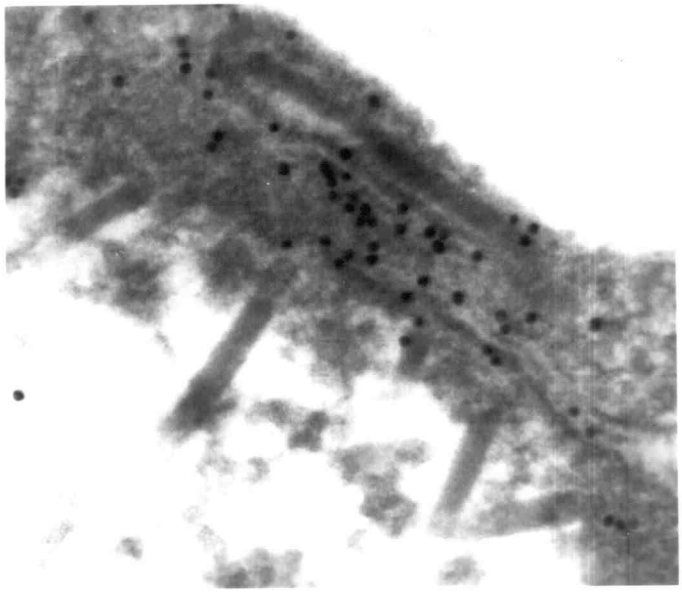
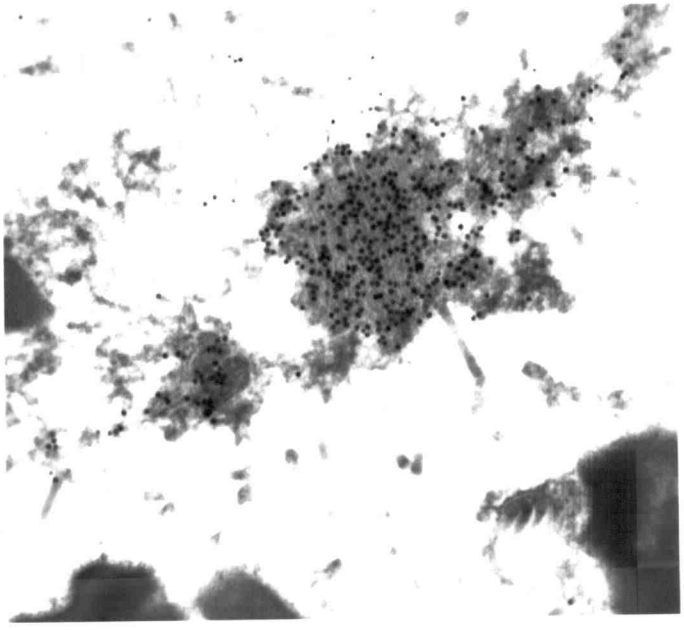


Fig. 4.4

c)



d)

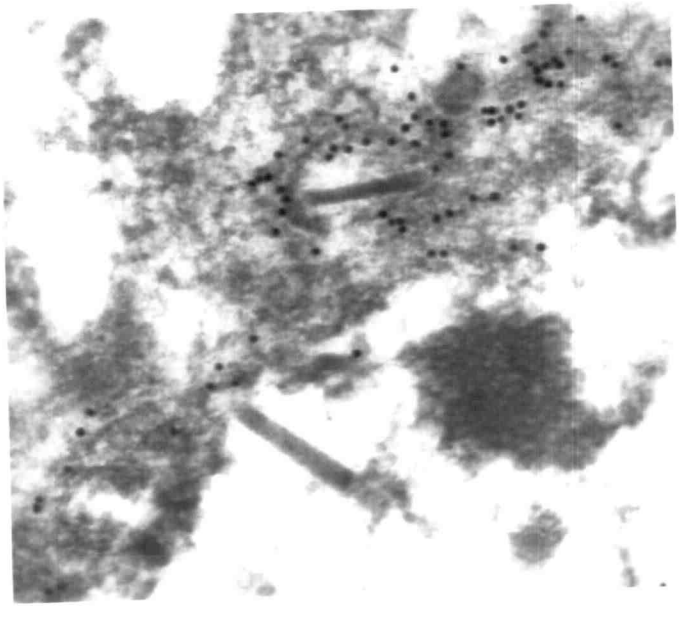


Fig. 4.4 continued

e)

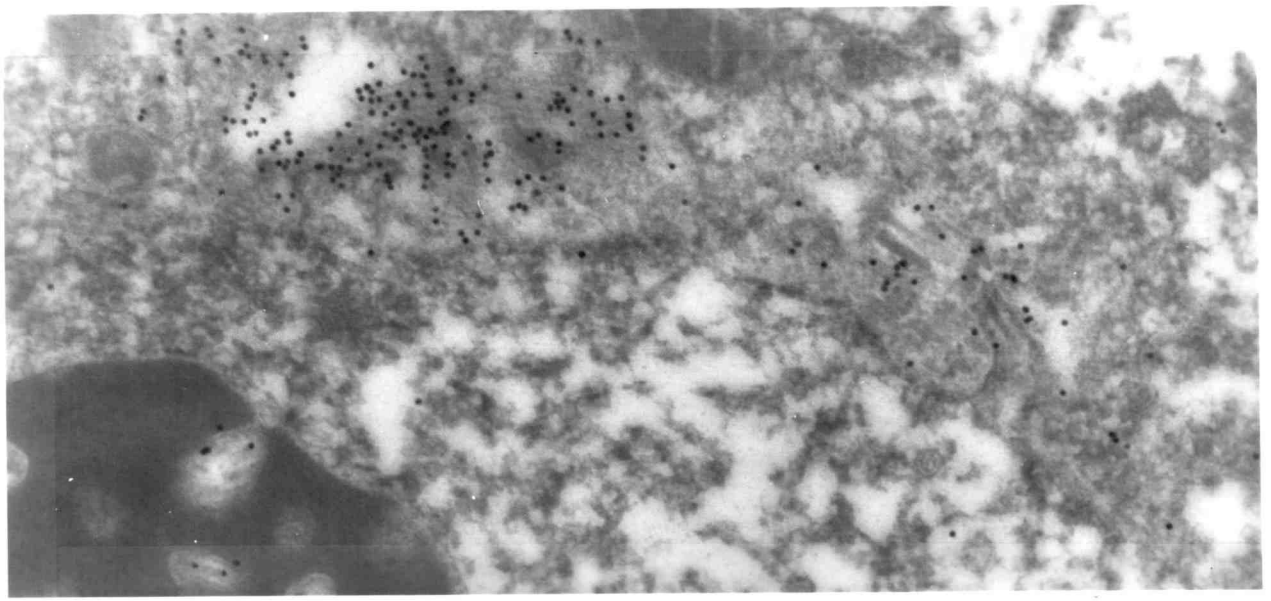


Fig. 4.4 continued

CHAPTER 5

The Spheroidin-like Protein from a Baculovirus of *Orgyia pseudotsugata* is Concentrated in Cytoplasmic Inclusion Bodies Late in Infection.

Christian H. Gross, Gregory M. Wolgamot, Rebecca L. Q. Russell, Margot N. Pearson and George F. Rohrmann

Department of Biochemistry and Biophysics
Oregon State University
Corvallis, Oregon 97331

Department of Agricultural Chemistry
Oregon State University
Corvallis, Oregon 97331

Abstract

The gene encoding the spheroidin-like protein of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) was located and sequenced. It was found to have 62 and 37% amino acid sequence identity with the spheroidin-like protein of the *Autographa californica* MNPV and spheroidin of the *Choristoneura biennis* entomopoxvirus, respectively. The mRNA start site of the OpMNPV spheroidin-like protein gene was mapped within a late promoter sequence (TTAAG). A *trp*E fusion protein containing 55% of the OpMNPV spheroidin-like protein gene was used to generate a monospecific antiserum. Western blot analysis of OpMNPV infected *Lymantria dispar* cells detected spheroidin-like protein expression beginning at 24 hours post infection. Immunofluorescence microscopy localized the spheroidin-like protein to intense foci surrounding the nucleus. Immunogold staining and electron microscopy indicated that the protein is concentrated in cytoplasmic inclusion bodies late in infection. The OpMNPV spheroidin-like protein was not associated with the polyhedron envelope of the occlusion bodies. In contrast, the AcMNPV spheroidin-like protein was associated with the matrix of the occlusion bodies and not with the polyhedron envelope.

Introduction

Nuclear polyhedrosis viruses (NPVs) are members of the *Baculoviridae*, a family of large double-stranded DNA viruses with a genomes of 88-160 kbp. They are pathogenic for arthropods, primarily insects of the orders Lepidoptera, Hymenoptera, and Diptera. NPVs replicate in the host cell nucleus and are characterized by an infection cycle that involves the production of two structurally distinct virion phenotypes. The budded virus (BV) phenotype, is produced from nucleocapsids which bud through and acquire their envelope from the host plasma membrane which has been modified by at least one virally encoded protein. Budded virus are responsible for the systemic infection of the insect host. In contrast, polyhedra derived virus (PDV) phenotype nucleocapsids are enveloped within the nucleus, presumably by *de novo* synthesis, and are then occluded in polyhedron-shaped crystals composed primarily of a protein called polyhedrin. Polyhedra are surrounded by an electron dense structure called the polyhedron envelope or polyhedron calyx. Polyhedra are stable for extended periods in the environment and function to spread the virus between insects.

Recently, Vialard et al., (1990) reported that the inclusion body protein (called spheroidin) of an entomopoxvirus pathogenic for *Choristoneura biennis* (CbEPV) shows over 39% amino acid sequence identity with an open reading frame (ORF) from the *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) that encodes a 37 kDa glycoprotein originally described by Wu and Miller (1989). Using antibodies against the CbEPV spheroidin, in conjunction with immunofluorescence microscopy and Western blot analysis, Vialard et al reported that the spheroidin-like protein (SLP) of AcMNPV appeared to be associated with the AcMNPV polyhedron envelope.

In this report, we describe investigations on the SLP from a related system. We located, sequenced, and transcriptionally mapped the SLP gene from the NPV of *Orgyia pseudotsugata* (OpMNPV). In

addition, a *trp*E-SLP fusion protein was constructed and used for the production of a monospecific antiserum. This antiserum was used for the characterization of the expression and immunocytochemical localization of the SLP in OpMNPV infected *Lymantria dispar* cells. Using the *trp*E-SLP antiserum we also localize SLP in AcMNPV infected *Spodoptera frugiperda* cells.

Materials and Methods

Virus, cells, and spheroidin antiserum

The cloned isolate of OpMNPV was described in Quant-Russell et al. (1987). AcMNPV polyhedra were provided by John Kuzio. *Lymantria dispar* (Ld-652Y) cells were propagated in T-flasks and using TNM-FH media (Summers and Smith, 1987) supplemented with 10% fetal bovine serum (FBS). For production of the budded virus, *Lymantria dispar* cells that had been adapted to shaker culture (a gift from Stephan Weiss, Gibco Laboratories) were grown in TNM-FH-10%FBS supplemented with 0.1% Pluronic-F68 (Gibco Laboratories). The CbEPV spheroidin antiserum was the gift of Jorge E. Vialard.

Purification of virus

For studies involving infected cells or budded virus, cells were infected with OpMNPV BV at a multiplicity of infection (moi) of 10 following the procedures of Bradford et al. (1990) unless otherwise indicated. For Western blot analysis, BV was isolated from two 150 ml shaker cultures of OpMNPV-infected *Lymantria dispar* cells at 72 hrs.p.i.. After a low speed centrifugation to remove the cells (2000xg for 10 min), the supernatant was centrifuged at 80,000xg for 30 min. This preparation was resuspended in 3 ml of TE (10 mM Tris (pH 7.5), 1 mM EDTA) and is called the infected-cell supernatant in this report. Samples of this preparation were further purified by centrifugation through a 38-52% sucrose gradient at 80,000xg for 2 hr at 4 ^{o}C, the virion band was removed, diluted in TE (10 mM Tris pH 7.5, 1 mM EDTA), and sedimented at 80,000xg for 1 hr to remove sucrose. Viral occlusion bodies were isolated from infected *Orgyia pseudotsugata* larval cadavers as previously described (Rohrmann et al., 1978).

Cloning and DNA sequencing

All cloning employed pBlueScribe (pBS) (Stratagene) maintained in *E. coli* JM 83 cells using standard methods (Maniatis, 1982). Double-

stranded plasmid DNAs were sequenced by using [^{35}S] dATP and Taq polymerase (Promega) by the dideoxy chain termination method of Sanger et al. (1977) as modified by Toneguzzo et al. (1988). Exonuclease III deletion mutants were produced by using the method of Henikoff (1987). The SLP gene was sequenced in both directions.

RNA isolation and transcriptional mapping

Total Cell RNA for use in primer extension and S1 nuclease analyses were isolated from OpMNPV infected *Lymantria dispar* cells at various times post infection by a CsCl centrifugation method similar to Glisin et al. (1974) (for modifications see chapter 2). For identification of the SLP transcription start sites, an 18-mer oligonucleotide complementary to a sequence near the 5' end (nt 448 to 465, Fig 2) of the SLP open reading frame was synthesized and used for primer extension analysis (Fig. 5.2). The synthetic oligonucleotide was 5' end-labeled as follows: To 100 ng of the primer was added 50 µCi [γ-^{32}P]ATP (6000 Ci/mmol) (New England Nuclear), 2 µl of 10X kinase buffer (500 mM Tris (pH 7.4), 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine), 7 units of T4 polynucleotide kinase, and incubated at 37 ^{o}C for 30 min in a 20 µl volume. The labeled oligonucleotide (5 ng) was annealed to 40 µg of total cell RNA at 50 ^{o}C for 45 min in a total volume of 15 µl (10X annealing buffer: 2.5 M KCl and 100 mM Tris, pH 8.3). To each annealing reaction, 24 µl H_2O, 4.5 µl 10 X primer extension buffer [225 mM Tris, (pH 8.3), 400 mM KCl, 50 mM $MgCl_2$, 50 mM DTT, and 2.5 mM dNTP's] and 300 units M-MLV reverse transcriptase (Bethesda Research Laboratories) was added and incubated for 30 min at 50 ^{o}C. Five µl of 3 M NaAc (pH 5.2) and 100 µl of ethanol were added and the nuclei acids precipitated at - 20 ^{o}C, resuspended in 6 µl of 0.1 M NaOH, 1 mM EDTA and incubated at 30 ^{o}C for 30 min to hydrolize the RNA. An equal volume of 95% formamide containing 0.05% bromophenol blue and 0.05% xylene cyanol dyes was added, the solutions were heated at 80 ^{o}C for 3 min, and electrophoresed on 8% polyacrylamide, 7 M urea gels. For S1 nuclease mapping of the 3' end of the SLP transcripts, 30 µg of each RNA sample was annealed to a 3'

end-labeled fragment. Annealing, hybridization, and S1 nuclease digestion were performed as described by Favaloro et al. (1980).

SLP antibody production: construction, expression, and isolation of the *trp*E-SLP fusion protein

The *trp*E-SLP gene fusion was constructed by digesting a plasmid (pBlueScribe - pBS, Stratagene) containing 1.6 kb Kpn I-Pst I restriction fragment (Fig. 5.1) with EcoR I and Hind III. The EcoR I-Hind III fragment was subcloned into the *trp* E expression vector pATH 1 (Koerner et al., 1991) to construct an in-frame gene fusion containing 175 amino acids (≈20 kDa) of the C-terminus of the SLP ORF. Recombinant plasmids were transformed into HB101 and grown in modified M9 medium as previously described (Gross and Rohrmann, 1990). For isolation of fusion protein, 100 ml cultures of bacteria was centrifuged and resuspended in 3 ml of 2 X SDS-PAGE buffer (0.125 M Tris (pH 7.5), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% Bromophenol blue) and boiled for 5 min. Half the sample (1.5 ml) was electrophoresed on a 20 cm long 1.5 mm thick 10% SDS-polyacrylamide gel in a Protean II (Bio-Rad) electrophoresis unit at 35 volts for 12-15 hours. The fusion protein band was identified by cutting three strips out of the gel (one on each margin and one in the center) and staining with Coomassie brilliant blue. The SLP fusion band was identified, excised, and electroeluted at 100 V overnight using a Schleicher and Schuell Elutrap apparatus. The amount of protein in the eluate was determined by Bradford protein assay (BioRad). A New Zealand White female rabbit was initially injected with approximately 500 µg of fusion protein emulsified in complete Freund's adjuvant. Subsequent biweekly injections of approximately 200 µg of fusion protein emulsified in incomplete Freund's adjuvant were given starting the third week after initial injection. Immune serum was collected 7 days after the third boost. Preimmune serum was collected before the initial injection.

Western blots analysis

For analyis of the expression of the SLP in infected cells, *Lymantria dispar* cells from different times post infection were lysed in 2X SDS-PAGE sample buffer and an amount of protein equivalent to approximately 4.8 X 10^4 cells was electrophoresed on each lane of a 10% SDS-polyacrylamide gel (Laemmli, 1970). Western blots were prepared as previously described (Quant-Russell et al., 1987). The *trp*E-SLP antiserum was diluted 1:1000 in TBS-T (20 mM Tris (pH 7.5), 500 mM NaCl, 0.05% Tween 20) and incubated on Western blots for 2 hours. Blots were rinsed in three 10 min washes of TBS-T and incubated 1-2 hours in a 1:7500 dilution of goat anti-mouse IgG conjugated with alkaline phosphatase (Promega). Blots were rinsed (three times for 10 min) in TBS-T. Immunoreactive proteins were detected by incubating the blot in a substrate solution of 330 µg/ml nitro blue tetrazolium and 165µg/ml 5-bromo-4-chloro-3 indolyl phosphate.

N-linked glycosylation assay

To analyze for N-glycosylation, *Lymantria dispar* cells were infected at an m.o.i. of 10 and grown in TNM-FH-10% FBS medium containing 10 µg of tunicamycin per ml prepared from a 1mg/ml stock in dimethyl sulfoxide. Cells were collected 60 hrs.p.i. in electrophoresis sample buffer, separated by SDS-PAGE and Western blot analysis was performed

Immunofluorescence microscopy

Immunofluorescence microscopy studies of OpMNPV-infected cells were performed as previously described (Pearson et al., 1988). Cell were infected at an m.o.i. of 100. The *trp*E-SLP antiserum was used at a dilution of 1:1000 in buffer A.

Immunoelectron microscopy

Lymantria dispar cells were infected with OpMNPV at an m.o.i. of 100. The cells were harvested and fixed in 2.5% glutaraldehyde, dehydrated with ethanol, and embedded in LR White resin (Russell and Rohrmann, 1990a). The *trp*E-SLP antiserum was used at a dilution of 1:2000. Immunogold staining was carried out as previously described using 10 nm gold particles (Russell and Rohrmann, 1990a).

Results

Nucleotide sequence of the OpMNPV SLP and similarity to AcMNPV SLP and CbEPV spheroidin

The SLP gene of AcMNPV is located downstream of the DNA polymerase gene (Wu and Miller, 1989). Because of the colinearity of the OpMNPV and AcMNPV genome (Leisy et al., 1984), a region downstream of the OpMNPV DNA polymerase gene was cloned and sequenced. An ORF encoding the carboxy terminal 125 amino acids of the DNA polymerase gene from (Fig. 5.2, nt 1 to 374) was shown to have 54% amino acid sequence identity to the corresponding region of the AcMNPV DNA polymerase gene. The complete SLP gene (Fig. 5.2, 401 to 1383) encoding a protein of 321 amino acids with a predicted MW of 36.1 kDa, was identified immediately downstream of the DNA polymerase gene. The OpMNPV SLP protein has 62.3% and 36.6% amino acid sequence similarity with AcMNPV SLP and CbEPV spheroidin protein, respectively (Fig. 5.3). Five conserved regions previously identified by Vialard et al. (1990) are also conserved in the OpMNPV SLP protein (Fig. 5.3) and except for region 2 show 80% or greater identity between the three proteins.

Transcriptional mapping and temporal expression of the SLP gene

To locate the transcriptional start site and the temporal expression of mRNA from the SLP gene, a 5' end-labeled oligonucleotide primer was annealed to total RNA isolated from *Lymantria dispar* cells at various times p. i. and elongated with reverse transcriptase. The size of the primer extension products were 58 and 59 nt as determined by comparison with a DNA sequencing ladder (Fig. 5.4). These products mapped to 13 and 14 nucleotides upstream of the SLP open reading frame and were located at positions 3 and 4 (nt 407 and 408, Fig. 5.2) in the sequence, TTAAG. The earliest primer extension products were detected at 24 hrs.p.i. and mRNA was present to 72 hrs.p.i., the last time point assayed (Fig. 5.4).

To map the 3' end of the SLP gene, S1 nuclease analyses were performed using a 1.6 kb Xho I-Pst I fragment (Fig. 5.1) that overlaps the 3' end of the gene. The fragment was 3' end-labeled at the Xho I site and annealed to RNA isolated from 36-72 hrs.p.i. (Fig. 5.5). The major protected fragment was approximately 1.3 kb which indicates that the 3' end of the gene is located approximately 890 nucleotides downstream of the SLP translational termination and maps to a region not sequenced (Fig. 5.1). Two other protected fragments were approximately 470 and 605 bp were also detected (Fig. 5.5) that map to 60 and 190 nucleotides respectively downstream of the SLP translational termination, a region not sequenced.

Characterization of SLP expression and N-linked glycosylation

To generate antibodies specific to the SLP, a gene fusion was constructed in a pATH 1 bacterial expression plasmid (see methods) (Fig. 5.2). The *trp*E-SLP fusion protein was induced, purified, and used for antiserum production. The expression of the SLP in OpMNPV infected *Lymantria dispar* cells was investigated by reacting Western blots of cell extracts from various times post infection with *trp*E-SLP antiserum. The SLP was first detected at 24 hrs.p.i. and increased through 120 hrs.p.i., the last timepoint assayed (Fig. 5.6a). Preparations of BV and occluded virus (OV) were also subject to Western blot analysis. Although the SLP was present in material derived from infected cell supernatants (lane 1, Fig. 5.6b), it was not present in sucrose gradient-purified BV or OV preparations (lanes 2 and 3, Fig. 5.6b). A positive control used in this analysis was an antiserum against virion associated protein, p24, which was observed in all three lanes. An identical Western blot of the sucrose gradient purified OpMNPV BV or OV preparations failed to react with the CbEPV spheroidin antiserum described by Vialard et al, (1990) (data not shown).

Western blots of AcMNPV occlusion bodies produced in tissue culture and a time course of extracts from AcMNPV infected *Spodoptera frugiperda* cells were reacted with the *trp*E-SLP and

spheroidin antisera. Both antisera identified bands of 32 kDa (presumably the unglycosylated form of SLP), 37 kDa SLP (presumably the glycosylated form) and an apparent SLP dimer of 61 kDa in both occlusion bodies and infected cell extracts from 36 hrs. p.i. to 120 hrs. p.i. the last time point assayed (data not shown). Although the cause of the molecular weight difference between the monomer and dimer was unclear, the suspected SLP dimer was always correlated with the expression of the 37 kDa SLP monomer. In CbEPV, Western blots using the spheroidin antiserum, demonstrated a similar dimer band which was shown to be composed of the monomer by its partial protease digestion pattern (Yuen et al., 1990). In OpMNPV, an SLP dimer was not observed (Figs. 5.6a and b). To further examine the location of SLP in AcMNPV preparations, occlusion bodies were solubilized under several different conditions as described by Whitt and Manning (1988). Initially AcMNPV polyhedra were dissolved in dilute alkaline saline (DAS) and centrifuged at 12,000xg for 10 min. This pellet contains polyhedron envelopes and entrapped virions. The resulting pellet was resuspended in 100 µl of TE (10 mM Tris (pH 7.5), 1 mM EDTA) and incubated with 0.5% SDS or SDS plus 1% 2-mercaptoethanol and then centrifuged at 12,000xg. SDS treatment dissociates virions, but leaves the polyhedron envelope protein component of the polyhedron envelope intact (Whitt and Manning, 1988; Gombart et al., 1989). The SDS plus 2-mercaptoethanol treatment dissociates all components of the virions and polyhedron envelopes. Western blot analysis indicated that the 37 kDa and 61 kDa SLP were present in the DAS supernatant and pellet fractions, respectively. In addition, the 61 kDa protein was present in the SDS and SDS/2-mercaptoethanol supernatant fractions. If SLP behaves like the polyhedron envelope protein, one would expect SLP to not be released by the SDS treatment (data not shown). The appearance of the 37 kDa SLP in the DAS supernatant suggests that the SLP is present in the occlusion body matrix or in the PDV envelope region. We have observed that alkali treatment also results in the removal of the PDV envelope region (Russell and Rohrmann, 1990). In contrast, the 61 kDa band required SDS for its release, which suggests a different association in the occlusion bodies.

N-linked glycosylation of the OpSLP was examined by Western blot analysis using the *trp*E-SLP antiserum and 60 hr.p.i. infected cell extracts grown with and without tunicamycin. The faster mobility of the tunicamycin treated SLP band (Fig. 5.6c, +Tun) when compared to the untreated band (Fig. 5.6c, -Tun) indicates that OpMNPV SLP is N-glycosylated.

Immunocytochemical localization of the SLP

Immunofluorescence microscopy studies were conducted using the *trp*E-SLP antiserum to trace the expression and location of the SLP in OpMNPV infected *Lymantria dispar* cells. Anti-*trp*E (an antiserum produced against the non fused truncated *trp*E protein) (Gombart et al., 1989b) and preimmune serum controls showed no staining of infected cells (data not shown). Although only minimal staining of uninfected cells was observed (Fig. 5.7a). In 76 hrs. p.i. cells, the SLP was concentrated in foci of intense cytoplasmic staining (Fig. 5.7b). This staining also appeared to show a polar orientation such that one side of the cells seemed to be distorted and stain relatively more intensely than the rest of the cell.

To more precisely localize the SLP protein, OpMNPV-infected *Lymantria dispar* cells were examined by immunogold staining and electron microscopy. Intense staining of SLP was localized to cytoplasmic inclusion bodies late in infection (Fig. 5.8a-b). These inclusion bodies were normally located throughout the cytoplasm. No significant staining of the polyhedra was seen at 84 hrs.p.i. (Fig. 5.8c) or at other times in the infection. However, in AcMNPV infected *Spodoptera frugiperda* cells the SLP protein was localized to occlusion bodies by immunogold labeling using the *trp*E-SLP antiserum (Fig. 5.8d). The staining was found distributed throughout the occlusion matrix and near the embedded virions. The AcMNPV polyhedron envelope structure did not specifically stain. In AcMNPV, cytoplasmic inclusion bodies similar to the ones seen in OpMNPV were found but immunostained poorly (data not shown). Neither uninfected nor

infected cells showed significant staining with *trp*E-SLP or anti-*trp*E serum control antisera, respectively (data not shown).

Discussion

The OpMNPV SLP gene is located downstream of the DNA polymerase gene, which is the same location as the AcMNPV SLP gene (Wu and Miller,1989). The predicted OpSLP amino acid sequence shows 37% and 62% similarity with CbEPV spheroidin and AcMNPV SLP, respectively. Strong conservation of the these genes was in the core of the protein (73%), the N- and C- terminal regions (13 and 51 amino acids) demonstrated lower amounts of similarity (36% and 18%). The SLP of OpMNPV was shown to be N-glycosylated and the amino acid sequence has one N-linked glycosylation site at amino acid 192, which is conserved amoung the three proteins (Fig. 5.3). The spheroidin and AcMNPV SLP each has an additional N-linked glycosylation site at amino acids 176 and 293, respectively (Fig. 5.3).

The 5' transcriptional start site was mapped within a late promoter consensus sequence 13 and 14 nucleotides upstream of the SLP ATG and mRNA specific to the SLP ORF was detected starting at 24 hrs.p.i. The SLP mRNA start site was located at the 3rd and 4th nucleotides of TTAAG. Although late promoters most often have the sequence A/GTAAG (Rohrmann, 1986), TTAAG has also been reported for the AcMNPV 35 K gene (Nissen and Friesen, 1989). In contrast to OpMNPV SLP, the AcMNPV SLP 5' transcription start sites mapped to two late promoter elements at 11 and 87 nucleotides (GTAAG and ATAAG) upstream of the translational start site (Wu and Miller,1989). The 3' transcription termination sites in OpMNPV were approximately 60, 190, and 890 nucleotides downstream of the translational termination codon. In AcMNPV, three transcription termination sites were also found approximately 30, 180, and 510 nucleotides downstream of the translational termination codon (Wu and Miller, 1989).

Western blot analysis using a polyclonal antiserum produced against a *trp*E-SLP fusion protein indicated that the SLP was expressed by 24 hrs.p.i. and was not detected associated with the OpMNPV BV or occlusion bodies (OB). These results differ from Vialard et al. (1990)

who demonstrated the presence of the SLP in purified AcMNPV occlusion bodies by Western blot analysis. We re-examined their results by reacting the *trp*E-SLP and spheroidin antisera (provided by Vialard) to Western blots of purified AcMNPV occlusion bodies. We found that both antisera react with a 37 kDa SLP, which confirmed the presence of the SLP associated with AcMNPV occlusion bodies as reported by Vialard et al. (1990).

Immunofluorescence microscopy showed that SLP was concentrated in foci of intense staining in the cytoplasm of OpMNPV infected *Lymantria dispar* cells. Immunoelectron microscopy demonstrated that the OpSLP, was highly concentrated in cytoplasmic inclusion bodies late in infection. The almond moth (*Cadra cautella*) and European fir budworm (*Choristoneura murinana*) infected with NPV's have been reported to have cytoplasmic inclusions or protein crystals (Adams and Wilcox, 1968; Huger and Kreig, 1968). Whether these inclusion are composed of SLP is unknown. In contrast, in AcMNPV SLP was located to the occlusion bodies matrix using the *trp*E-SLP antiserum and immunoelectron microscopy. The reason for the difference in SLP localization between the two viruses is unclear. No evidence indicated that the SLP was associated with polyhedron envelope structure of either OpMNPV or AcMNPV. Wu and Miller (1989) reported that they were unable to delete the SLP gene from AcMNPV genome which indicated an essential role in the infection cycle.

Despite the difference in infection cycle and promoter structure of the two types of virus (baculoviruses replicate in the nucleus whereas entomopox virus replicate in the cytoplasm), it is intriguing to speculate that the CbEPV speroidin gene was derived from a coinfection with a baculovirus. While this theory is highly speculative, it has recently been demonstrated that another entomopox virus has a spheroidin gene completely different from the CbEPV gene. This suggests that entomopox virus spheroidin may have an number of independent lineages. This is in contrast to other genes identified in entomopox viruses which are highly conserved with their

homologues in orthopox viruses (Hall and Moyer, 1991; Banville et al., 1992).

Fig. 5.1 Location of the SLP gene on the OpMNPV genome. The Hind III map of the OpMNPV genome is shown at the top and the positions of cosmid clones 47,1,54,58, and 39 are indicated. The locations of the polyhedrin (PH), DNA polymerase (DNA pol), p39 capsid protein (capsid), budded virus envelope glycoprotein (gp64), polyhedron envelope protein (PEP), and the p10 protein are shown below the Hind III map. The expanded restriction map below the genome shows the location of the SLP gene along with prominent restriction enzyme sites. The location of the SLP mRNA is also indicated. The shaded box indicates the area sequenced. The location of the open reading frame encoding the SLP is shown in relation to the DNA polymerase gene.

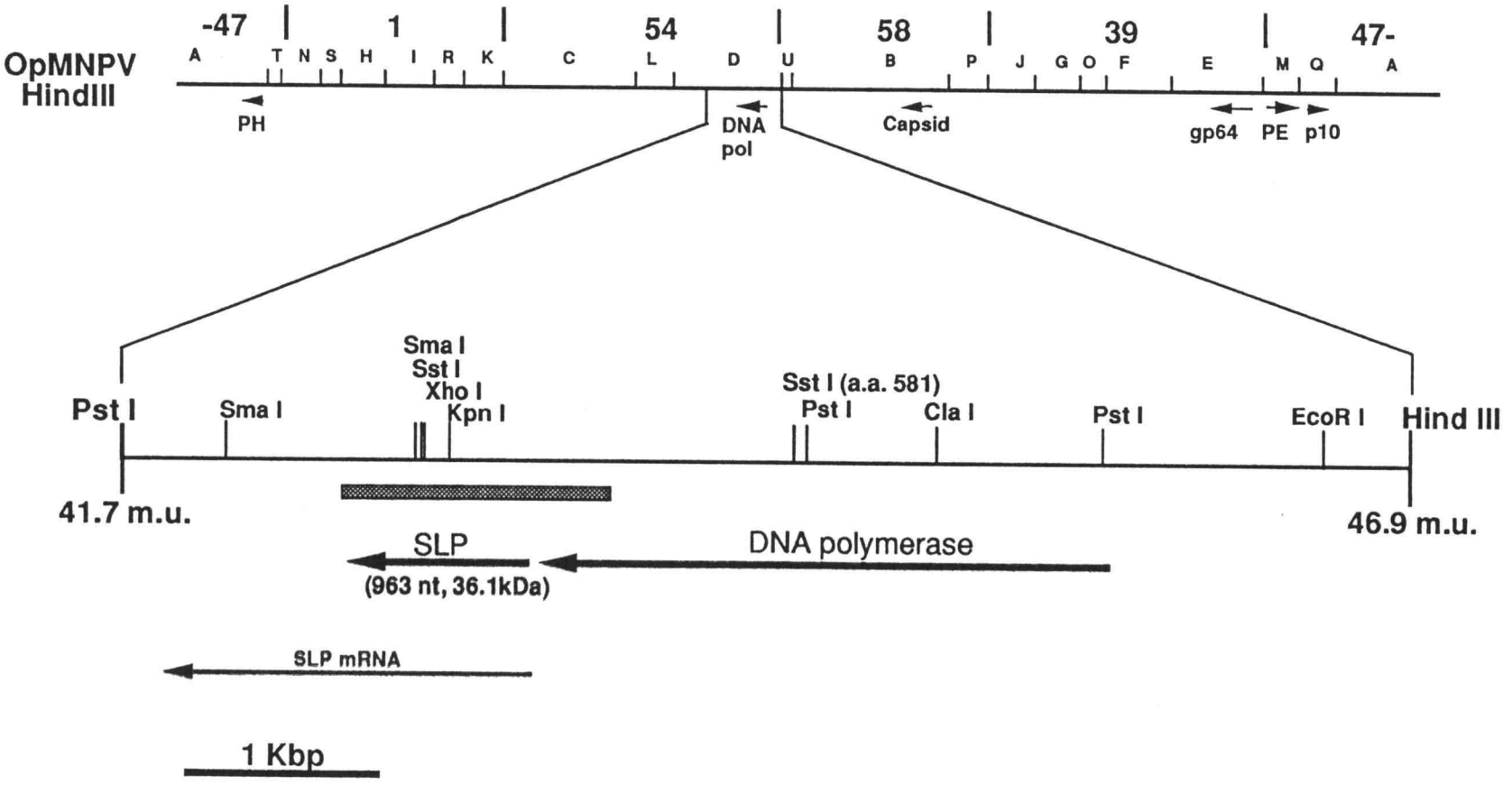

OpMNPV
HindIII
-47
1
54
58
39
47-
A
T N S H I R K
C
L
D
U
B
P
J
G O F
E
M Q
A
PH
DNA pol
Capsid
gp64
PE
p10
Sma I
Sst I
Xho I
Kpn I
Pst I
Sma I
Sst I (a.a. 581)
Pst I
Cla I
Pst I
EcoR I
Hind III
41.7 m.u.
46.9 m.u.
SLP
(963 nt, 36.1kDa)
DNA polymerase
SLP mRNA
1 Kbp

Fig. 5.1

Fig. 5.2 Nucleotide sequence of the OpMNPV SLP gene region. The amino acid sequence predicted from DNA polymerase (nt 1 to 374) and SLP genes (nt 401 to 1383) is shown below the nucleotide sequence. The location of the 18-mer oligonucleotide primer used for primer extension analysis is shown above the sequence (primer) and the SLP mRNA initiation site is indicated. The location of major restriction sites including the Kpn I site used for the production of the SLP-*trp*E gene fusion protein are indicated. Numbers to the right of the sequence indicate the position of nucleotides and amino acids, respectively.

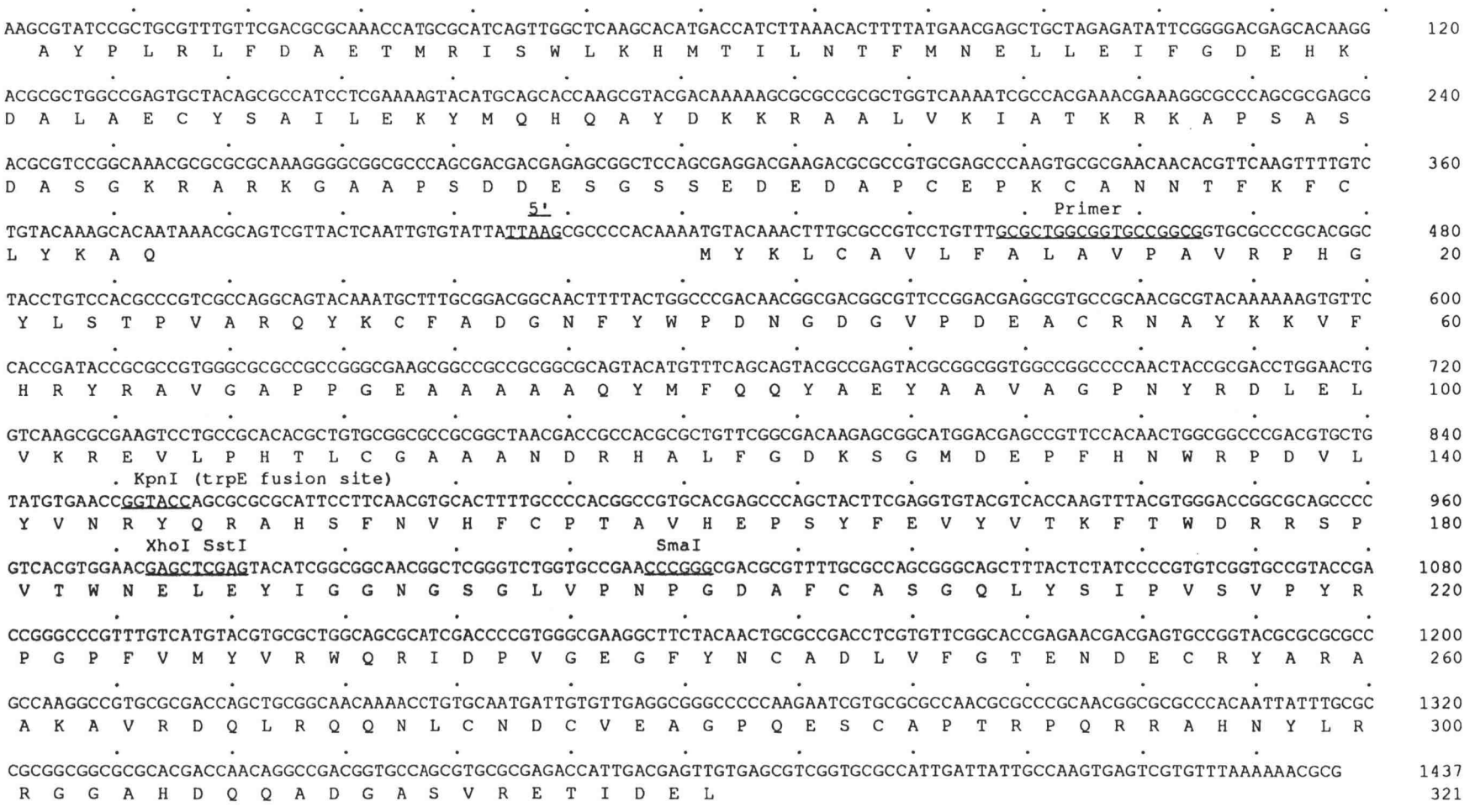

AAGCGTATCCGCTGCGTTTGTTCGACGCGCAAACCATGCGCATCAGTTGGCTCAAGCACATGACCATCTTAAACACTTTTATGAACGAGCTGCTAGAGATATTCGGGGACGAGCACAAGG 120
A Y P L R L F D A E T M R I S W L K H M T I L N T F M N E L L E I F G D E H K
ACGCGCTGGCCGAGTGCTACAGCGCCATCCTCGAAAAGTACATGCAGCACCAAGCGTACGACAAAAAGCGCGCCGCGCTGGTCAAAATCGCCACGAAACGAAAGGCGCCCAGCGCGAGCG 240
D A L A E C Y S A I L E K Y M Q H Q A Y D K K R A A L V K I A T K R K A P S A S
ACGCGTCCGGCAAACGCGCGCGCAAAGGGGCGGCGCCCAGCGACGACGAGAGCGGCTCCAGCGAGGACGAAGACGCGCCGTGCGAGCCCAAGTGCGCGAACAACACGTTCAAGTTTTGTC 360
D A S G K R A R K G A A P S D D E S G S S E D E D A P C E P K C A N N T F K F C
5' Primer
TGTACAAAGCACAATAAACGCAGTCGTTACTCAATTGTGTATTATTAAGCGCCCCACAAAATGTACAAACTTTGCGCCGTCCTGTTTGCGCTGGCGGTGCCGGCGGTGCGCCCGCACGGC 480
L Y K A Q M Y K L C A V L F A L A V P A V R P H G 20
TACCTGTCCACGCCCGTCGCCAGGCAGTACAAATGCTTTGCGGACGGCAACTTTTACTGGCCCGACAACGGCGACGGCGTTCCGGACGAGGCGTGCCGCAACGCGTACAAAAAAGTGTTC 600
Y L S T P V A R Q Y K C F A D G N F Y W P D N G D G V P D E A C R N A Y K K V F 60
CACCGATACCGCGCCGTGGGCGCGCCGCCGGGCGAAGCGGCCGCCGCGGCGCAGTACATGTTTCAGCAGTACGCCGAGTACGCGGCGGTGGCCGGCCCCAACTACCGCGACCTGGAACTG 720
H R Y R A V G A P P G E A A A A A Q Y M F Q Q Y A E Y A A V A G P N Y R D L E L 100
GTCAAGCGCGAAGTCCTGCCGCACACGCTGTGCGGCGCCGCGGCTAACGACCGCCACGCGCTGTTCGGCGACAAGAGCGGCATGGACGAGCCGTTCCACAACTGGCGGCCCGACGTGCTG 840
V K R E V L P H T L C G A A A N D R H A L F G D K S G M D E P F H N W R P D V L 140
. KpnI (trpE fusion site)
TATGTGAACCGGTACCAGCGCGCGCATTCCTTCAACGTGCACTTTTGCCCCACGGCCGTGCACGAGCCCAGCTACTTCGAGGTGTACGTCACCAAGTTTACGTGGGACCGGCGCAGCCCC 960
Y V N R Y Q R A H S F N V H F C P T A V H E P S Y F E V Y V T K F T W D R R S P 180
. XhoI SstI SmaI
GTCACGTGGAACGAGCTCGAGTACATCGGCGGCAACGGCTCGGGTCTGGTGCCGAACCCGGGCGACGCGTTTTGCGCCAGCGGGCAGCTTTACTCTATCCCCGTGTCGGTGCCGTACCGA 1080
V T W N E L E Y I G G N G S G L V P N P G D A F C A S G Q L Y S I P V S V P Y R 220
CCGGGCCCGTTTGTCATGTACGTGCGCTGGCAGCGCATCGACCCCGTGGGCGAAGGCTTCTACAACTGCGCCGACCTCGTGTTCGGCACCGAGAACGACGAGTGCCGGTACGCGCGCGCC 1200
P G P F V M Y V R W Q R I D P V G E G F Y N C A D L V F G T E N D E C R Y A R A 260
GCCAAGGCCGTGCGCGACCAGCTGCGGCAACAAAACCTGTGCAATGATTGTGTTGAGGCGGGCCCCCAAGAATCGTGCGCGCCAACGCGCCCGCAACGGCGCGCCCACAATTATTTGCGC 1320
A K A V R D Q L R Q Q N L C N D C V E A G P Q E S C A P T R P Q R R A H N Y L R 300
CGCGGCGGCGCGCACGACCAACAGGCCGACGGTGCCAGCGTGCGCGAGACCATTGACGAGTTGTGAGCGTCGGTGCGCCATTGATTATTGCCAAGTGAGTCGTGTTTAAAAAACGCG 1437
R G G A H D Q Q A D G A S V R E T I D E L 321

Fig. 5.2

Fig. 5.3 Comparison of the predicted amino acid sequence from *Choristoneura biennis* EPV spheroidin, OpMNPV SLP, and AcMNPV SLP genes. The double dots indicate identical amino acids with respect to the OpSLP. Regions of high similarity identified by Vialard et al. (1991) are underlined and specified by number. The N-linked glycosylation sites for spheroidin (a.a. 176 and 196), OpSLP (a.a. 192), and AcSLP (a.a. 193 and 293) are underlined.

```
Spheroidin    1 MNKLILISL-IASLYQVEVDAHGYMTFPIARQRRCSAAGGNWYPVGGGGIQDPMCRAAYQN
                : ::    :  : :    :  :::   : :::  : : :    :  : :  :  :: ::
    OpMNPV    1 MYKL-CAVLF-A-LAVPAVRPHGYLSTPVARQYKCFADGNFYWPDNGDGVPDEACRNAYKK
                :  :    :: :    :::: ::::: : :::::: :::::::::::  :: :::::::
    AcMNPV    1 MIAL-LIALFAA-IHAPAVRSHGYLSVPTARQYKCFKDGNFYWPDNGDNIPDAACRNAYKS

Spheroidin   61 VFNKVLNSNGGDVIDASEAANYMYTQDNEYAALAGPDYTNICHIQQRVVPSYLCAAGASDW
                ::       :     :  :: ::  :  :::: ::: :         : :  :: : : :
    OpMNPV   59 VF-HRYRAVGAPPGEAAAAAQYMFQQYAEYAAVAGPNYRDLELVKREVLPHTLCGAAAND-
                :    :::     : ::  :::::::: :::::::::: :  : :  : :::::::  ::
    AcMNPV   60 V-YYKYRALDLESGAAASTAQYMFQQYMEYAAVAGPNYDDFDLIKQRVVPHTLCGAGSND-
                                              1                2

Spheroidin  122 SIRPFGDKSGMDLPGSWTPTIIQLSDNQQSNVVMELEFCPTAVHDPSYYEVYITNPSFNVY
                   :::::::: :          :  :        ::::::: ::: ::: :
    OpMNPV  118 RHALFGDKSGMDEPFHNWRPDVLYVDRYQRAHSFNVHFCPTAVHEPSYFEVYVT-KFTWDR
                :  :::::::::: ::::  ::  :::     :::::::: :::::::  : :  :::
    AcMNPV  119 RNSVFGDKSGMDEPFNNWRPNTLYLNRYQPVYQMNVHFCPTAIHEPSYFEVFIT-KSNWDR
                     3                                  4

Spheroidin  183 TDNVVWANLDLIYNNTVTLRPKLPESTCAANSMVYRFEVSIPVRPSQFVLYVRWQRIDPVG
                   : :  :  :  :  : :  :    :   :  :: : ::  :: ::::::::::::
    OpMNPV  178 RSPVTWNELEYIGGNGSGLVP-NPGDAFCASGQLYSIPVSVPYRPGPFVMYVRWQRIDPVG
                : : :::::::::::: : : : ::::  :    :::::  :::   ::::::::::::::
    AcMNPV  179 RNPITWNELEYIGGNDSNLIP-NPGDSLCDNSLVYSIPVVIPYRSNQFVMYVRWQRIDPVG
                                                                      5

Spheroidin  244 EGFYNCVDMKFKYSEGPDEEDIIEPEYEVDNEAECFAYRTNSGNVNVNPLQENKYMAYANK
                ::::::  :  :   :  ::          :           :   :   : ::    :
    OpMNPV  238 EGFYNCADLVF-GTE-NDECRYARAAKAV-RDQLRQQNLCN-DCVEAGP-QE--SCA-PTR
                ::::::::::  :   ::::::  :: : : :: :      :        :  ::    :
    AcMNPV  239 EGFYNCADLVF-ET-LDDECRYAQMAKVV-RSQL-Q--KHKLDARIDHNDEE--SC---WR

Spheroidin  305 AIRNINTHSNGCSRNRNNKNNYNKYYSKTYNYNQNRK
                  :    : :   : :
    OpMNPV  291 PQR--RAH-N-YLR-RGGAHDQQADGASVRETIDEL
                     :   : :              :
    AcMNPV  289 ----AR-KSN-Y--------------SSFFNPGF
```

Fig. 5.3

Fig. 5.4 5' transcriptional mapping of the SLP transcripts.
Transcription time course and location of the SLP transcription initiation site was determined by primer extension analysis. A 5' end-labeled oligonucleotide complementary to the 5' end of the SLP ORF (see Fig. 5.2) was annealed to total RNA isolated at various hours p.i. and elongated with reverse transcriptase. The size (nt) of the primer extension products are indicated on the left. Sizes of primer extension products were determined by alignment with a p1,2,PEPCAT sequence ladder (the p1,2,PEPCAT clone is described in Gross and Rohrmann, manuscript in prep.).

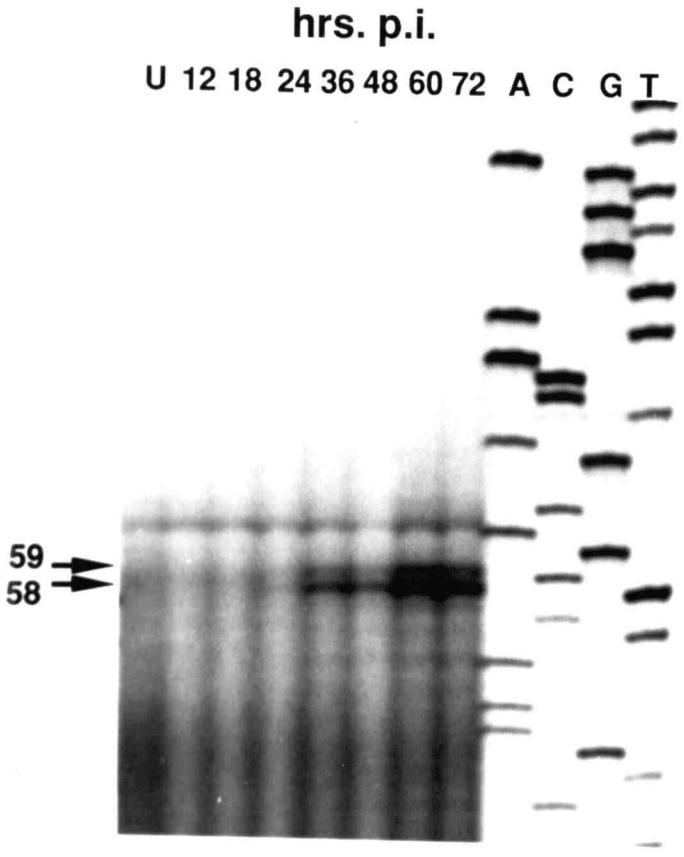


Fig. 5.4

Fig. 5.5 3' transcriptional mapping of the SLP transcripts. The location and transcription time course of the 3' ends of the SLP transcript was determined by S1 nuclease protection. A 1.6 kb Xho I-Pst I fragment was 3' end labeled with T4 DNA polymerase, hybridized to total RNA isolated at various hours p. i. and digested with S1 nuclease. The size of the S1 nuclease protected products are indicated on the left, as determined by a labeled 1 kb ladder (BRL) and a Hae III digested ϕx174 ladder (New England Biolabs)

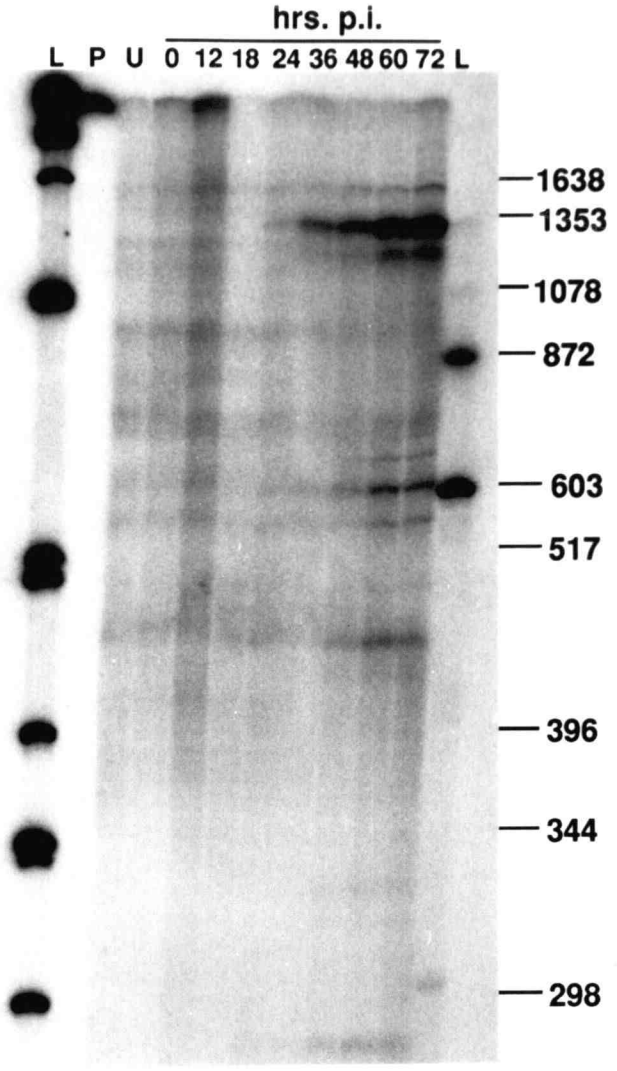

hrs. p.i.
L P U 0 12 18 24 36 48 60 72 L
1638
1353
1078
872
603
517
396
344
298

Fig. 5.5

Fig. 5.6 Western blot analysis of the SLP in OpMNPV infected *Lymantria dispar* cells and viral phenotypes. a) Expression of SLP in OpMNPV infected *Lymantria dispar* cells. The numbers on the top of each lane indicate hours post infection. Uninfected cells are represented by the letter U. b) Western blot analysis of the SLP in purified OpMNPV budded and occluded viral phenotypes. Control lane 1 infected cell supernatant (see purification of virus), lane 2 budded virus (BV), and lane 3 occluded virus (OV). c) Analysis of N-linked glycosylation of SLP in infected *Lymantria dispar* cells (see methods). The figure shows a Western blot analysis of the SLP treated without tunicamycin (-Tun) and with tunicamycin (+Tun). Sizes (kDa) of the protein standards are indicated on the left and the SLP is indicated on the right on all Western blots.

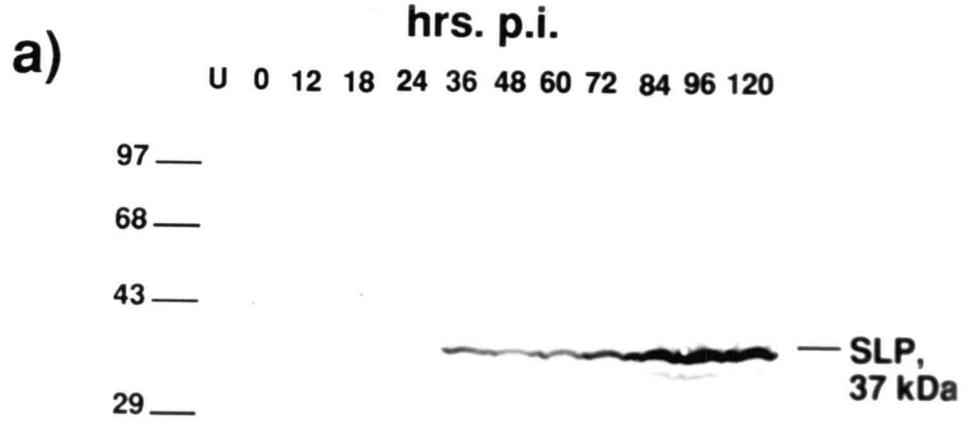
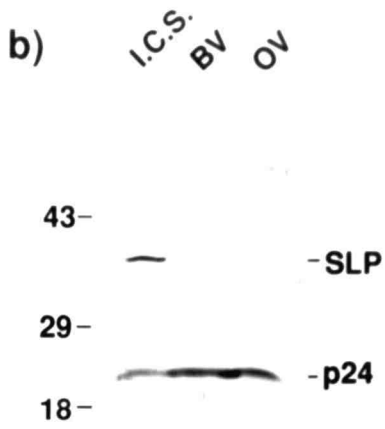


Fig. 5.6



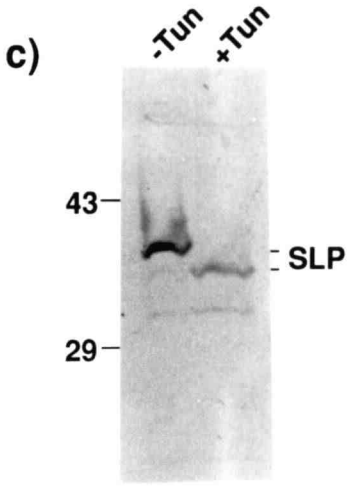
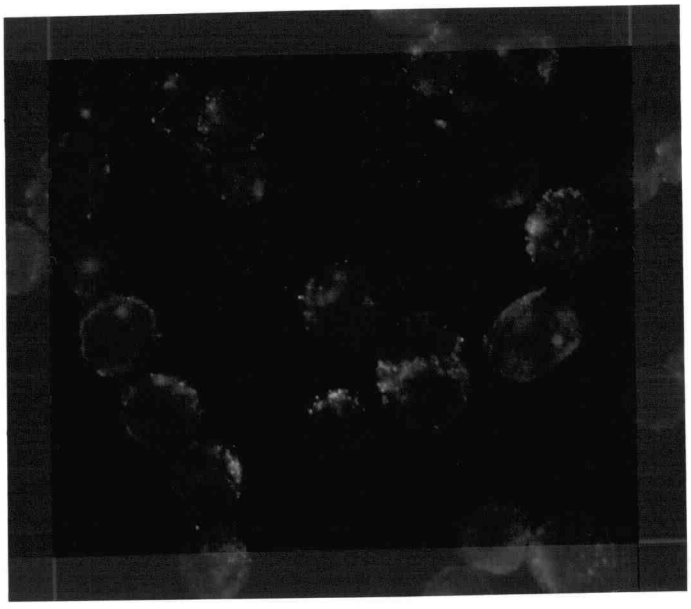


Fig. 5.6 continued

Fig. 5.7 Immunocytochemical localization of the SLP in OpMNPV infected *Lymantria dispar* cells. a) Indirect immunofluorescence staining of OpMNPV-infected *Lymantria dispar* cells at 76 hrs.p.i. b) Indirect immunofluorescence staining of uninfected *Lymantria dispar* cells. The antisera were diluted 1:1000.

a)



b)

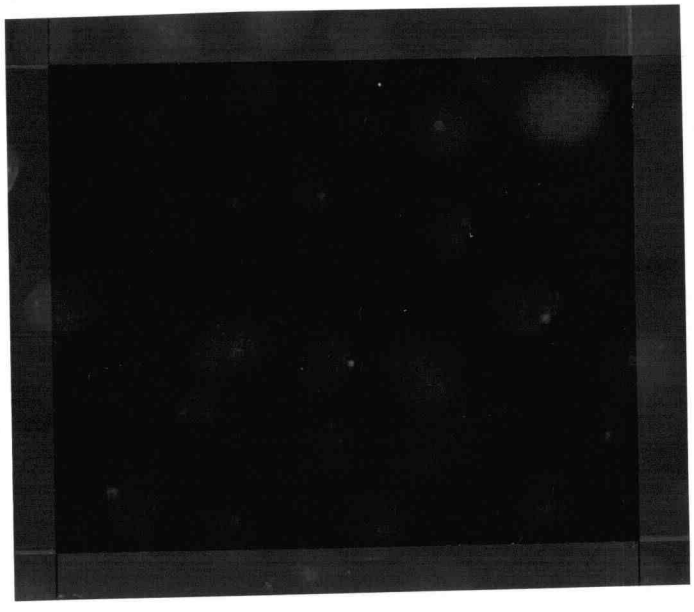
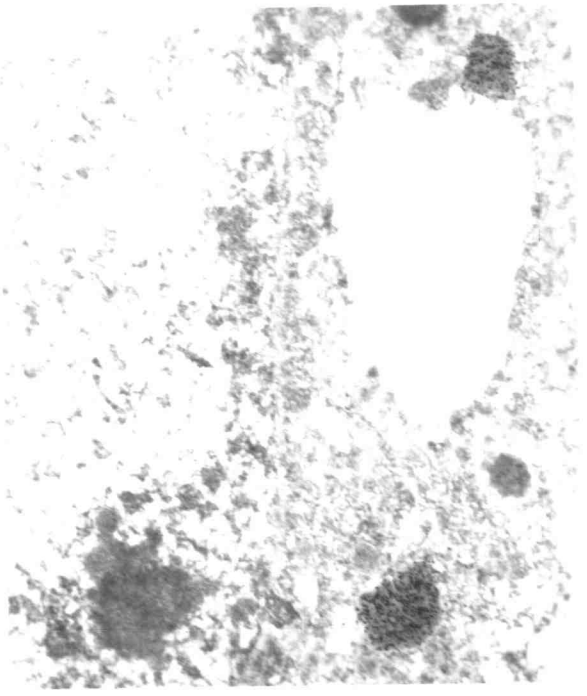


Fig. 5.7

Fig. 5.8 Immunogold staining of OpMNPV infected *Lymantria dispar* cells and AcMNPV infected *Spodoptera frugiperda* cells. a) Two SLP inclusion bodies in the cytoplasm of an OpMNPV infected *Lymantria dispar* cell (84 hrs.p.i.). b) SLP inclusion body in the cytoplasm of an OpMNPV infected *Lymantria dispar* cell (72 hrs.p.i.). c) Two OpMNPV occlusion bodies-immunogold labelled with anti-*trp*E-SLP. d) AcMNPV occlusion body-immunogold labelled with anti-*trp*E-SLP. The *trp*E-SLP antiserum was diluted 1:1000 (a) and 1:2000 (b-d). The scale: 1 cm is equal to a) 0.2 μM, b) 0.2 μM, c) 0.1 μM, d) 0.2 μM.

a)



b)

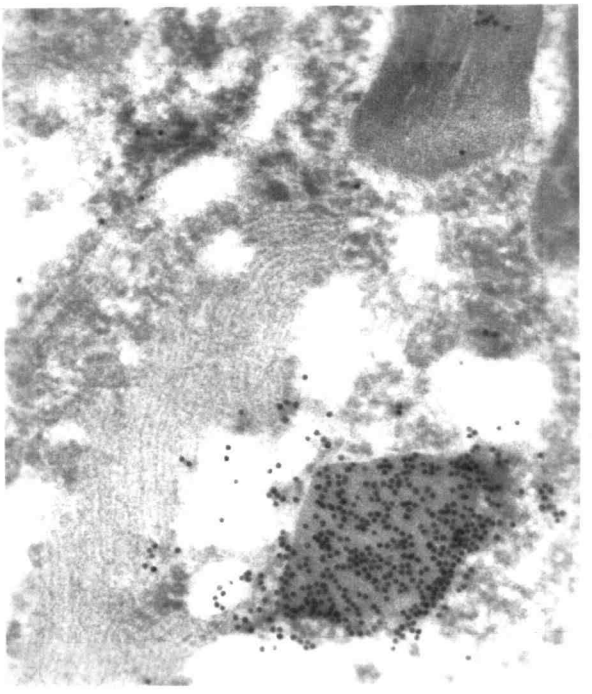


Fig. 5.8

c)

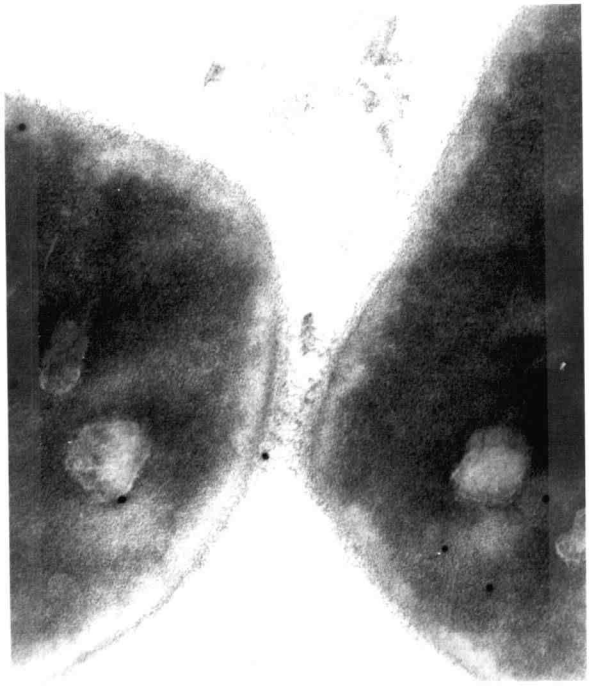


Fig. 5.8 continued

d)

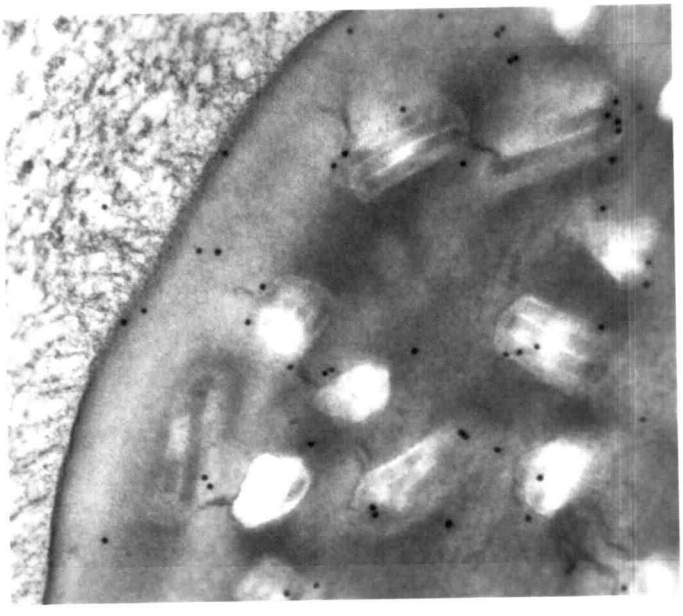


Fig. 5.8 continued

CHAPTER 6

Analysis of *Orgyia pseudotsugata* Multicapsid Nuclear Polyhedrosis Virus Mutants Lacking the p10 and Polyhedron Envelope Protein genes.

Christian H. Gross, Rebecca L. Q. Russell and George F. Rohrmann

Department of Biochemistry and Biophysics
Oregon State University
Corvallis, Oregon 97331

Department of Agricultural Chemistry
Oregon State University
Corvallis, Oregon 97331

Abstract

To investigate the role of the p10 and polyhedron envelope protein gene in polyhedron development, four deletion mutants were constructed of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. In the first two, the p10 gene was replaced with the β-glucuronidase or the β-galactosidase gene. In the third virus, the polyhedon envelope protein gene was replaced with the β-glucuronidase gene. In the fourth virus, both p10 and the polyhedron envelope protein genes were deleted and replaced with β-galactosidase and β-glucuronidase genes, respectively. Insects were infected with these recombinants and electron microscopy was used to examine morphological effects caused by the deletion of these genes. In cells from insects infected with the p10 minus virus, fibrillar structures were not observed and the polyhedron envelope seen in the wild type infection, was absent from polyhedron surfaces. However, immunogold labeling showed that the polyhedron envelope protein was localized around the polyhedra. In insects infected with the virus lacking the polyhedron envelope protein, a distinct polyhedron envelope was also not observed. However, immunogold labeling showed that the p10-fibrillar structures were still present. In insects infected with the recombinant lacking both the polyhedron envelope protein and p10, the polyhedra were found to have a distinct cubic morphology. In addition, the polyhedron envelope and the fibrillar structures were not observed.

Introduction

Nuclear polyhedrosis viruses (NPVs) are members of the *Baculoviridae* , which are a family of viruses pathogenic for arthropods, primarily insects of the orders Lepidoptera, Hymenoptera, and Diptera. The large (88-160 kb) double-stranded supercoiled DNA genomes of NPVs replicate in the host cell nucleus. NPVs are characterized by a complex infection cycle that involves the production of two structurally distinct virion phenotypes. The budded virus phenotype (BV), consists of nucleocapsids that acquire their envelope when they bud through the cytoplasmic membrane which has been modified by at least one viral encoded protein. This phenotype is responsible for systemic infection within the insect host. In contrast, the polyhedra-derived virus phenotype (PDV), is occluded in infected cell nuclei in polyhedron-shaped crystals composed primarily of a protein called polyhedrin. These polyhedra are surrounded by another envelope structure called the polyhedron envelope. The polyhedra are stable in the environment until ingested by insect larvae and thereby spreads the virus between insects.

A protein component of the baculovirus polyhedron envelope (called the polyhedron envelope protein or polyhedron calyx) was originally identified in the *Autographa californica* multicapsid NPV (Whitt and Manning, 1988) and subsequently the gene encoding this protein was identified and sequenced in both the *Orgyia pseudotsugata* multicapsid NPV (OpMNPV) (Gombart et al., 1989a) and AcMNPV (Gombart et al., 1989a; Oellig et al., 1987). Immunoelectron microscopy has shown the polyhedron envelope protein (PEP) to be an integral component of the polyhedron envelope (Russell and Rohrmann, 1990a; van Lent et al., 1990). In addition, PEP has been demonstrated to be a component of 'electron dense spacer' which are formed at the junctions of p10-containing fibrillar material (van Lent et al, 1990; Russell and Rohrmann, 1990a). Deletion of the p10 gene results in the absence of the fibrillar material and polyhedra lacking or with fragmented polyhedron envelopes (Williams et al., 1989). In addition, such polyhedra appear to be fragile and readily disintegrate (William et

al., 1989). It has also been demonstrated that polyhedra produced *in vitro* by an AcMNPV mutant lacking the PEP gene, did not have a polyhedron envelope (Zuidema et al., 1989). These observations have suggested that the proteins p10 and PEP interact and together play an important role in polyhedron morphogenesis

In this report, we describe electron microscopic investigations of insects infected with four recombinant OpMNPV's. In two of the recombinant viruses, the p10 gene was replaced with either a β-galactosidase (*lac* Z) or β-glucuronidase (GUS) gene. The PEP gene was replaced by a GUS gene in the third recombinant virus. The fourth virus was a double recombinant expressing *lac* Z in place of the p10 gene and GUS in place of the PEP gene. In addition, we examined the temporal expression of the p10 and PEP genes.

Materials and Methods

Virus, insect cells lines, insects, and antisera

The cloned isolate of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) was described in Quant-Russell et al. (1987). Virus stocks were titrated by endpoint dilution assays as described by Bradford et al. (1990). In case of titering the recombinant viruses, reporter gene activity was used to detect the presence of virus as described below. *Lymantria dispar* (Ld-652Y) cells were propagated in T-flasks and using TNM-FH media (Summers and Smith, 1987) supplemented with 10% fetal bovine serum (FBS). For production of budded virus, *Lymantria dispar* cells that had been adapted to shaker culture (a gift from Stephan Weiss, Gibco Laboratories) were grown in TNM-FH-10%FBS supplemented with 0.1% Pluronic-F68 (Gibco Laboratories). The *Orgyia pseudotsugata* fourth and fifth instar larvae were kindly provided by USDA. The recombinant budded viruses were injected into the insect larvae at approximately 50,000 $TCID_{50}$/insect. The p10 monoclonal antibody (Mab 210) and the polyhedron envelope protein polyclonal antiserum were used as described by Quant-Russell et al. (1987) and Gombart et al. (1989), respectively. The p39 monoclonal antibody (Mab 236) and the p24 polyclonal antiserum were used as described by Pearson et al. (1988) and Gross et al. (see Chapter 3), respectively.

Construction of p10 and polyhedron envelope protein gene transfer vectors

To construct the p10 transfer vector a 2.4 kbp Hind III-Sal I from Hind III Q was cloned into a pBlueScribe (pBS(-)) plasmid (Stratagene) using standard methods (Sambrook et al.,1989). The p10 containing plasmid was then digested with Xba I and Eco RI to remove restriction sites from the polylinker. The digested plasmid was then blunt ended with S1 nuclease religated and transformed in *E. coli* JM 83 cells. Site-directed mutagenesis was used to produce a unique Bam HI site three codons after the translational start site of the p10 gene (**ATG**TCCAAGCCC /**ATG**TCGGATCCC) by the method of Kunkel (1987). The primer used to create the mutation was (5'-

CTGCAATATCATC ATGTCGGATCCCAGCATTTTGACGC-3'). The modified p10 plasmid was digested with Bam HI and Sst I. A 2.1 kbp Bam HI-Sst I fragment containing the GUS gene was inserted, which resulted in a functional p10 promoter-GUS gene fusion (Fig. 6.1a). To construct a functional p10 promoter-*lac* Z gene fusion a 3899 bp Bam HI-SstI fragment which contained the *lac* Z gene was cloned into the modified p10 plasmid (Fig. 6.1b).

To construct a polyhedron envelope protein (PEP) gene transfer vector, we used a plasmid from another study (see Chapter 2) which contained the entire PEP gene region. This plasmid had a unique Bam HI site one codon after the PEP translational start site and a Sst I site at the 11th PEP codon were produced by site directed mutagenesis (see Chapter 2). The PEP construct was digested with Bam HI and Sst I and a 2.1 kbp Bam HI-Sst I fragment containing the GUS gene was inserted, which resulted in a functional PEP promoter-GUS gene fusion (Fig. 6.1c).

Construction and purification of recombinant viruses, using β-glucuronidase and β-galactosidase assays

To construct mutants lacking the p10, polyhedron envelope protein or both p10 and polyhedron envelope genes, *Lymantria dispar* cells were seeded at 1.5 x 10^6 cells per well in six-well tissue culture plates. Transfections of these cells using 1 μg of wild type OpMNPV DNA and 2 μg of a transfer plasmid were performed as described by Summers and Smith (1987). After 5 days, the supernatants containing mixtures of wild type and recombinant virus were collected and recombinant viruses were cloned by a limiting dilution protocol (Fung et al., 1988) using either a β-glucuronidase or β-galactosidase activity to identify a recombinant virus. β-glucuronidase was assayed by the method of Jefferson (1987) modified for small volumes. Supernatants from the recombination experiment were serially diluted in 96-well microtiter plates resulting in a final volume of 200 μl/well. Samples (120 μl) were removed and stored 4 °C in a new plate. The remaining 80 μl was frozen and thawed once and 150 μl of β-glucuronidase assay

buffer (Jefferson,1987) was added and incubated for several hours at 37 ^{o}C and then scored for fluorescence on a long UV (365 nm) transilluminator.

Cells infected with the recombinant virus containing the β-galactosidase gene were assayed for β-galactosidase by the method of Miller (1972) modified for small volumes. To 80 µl cell extracts prepared as described above, and 120 µl of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 0.27% 2-mercaptoethanol) was added. After incubation at 37 ^{o}C for five min, 40 µl of 4 mg/ml o-nitrophenyl-β-D-galactoside (ONPG) in 0.1 M $NaPO_4$ (pH 7.0) was added and incubated at 37 ^{o}C for several hours.

After the recombinant viruses had been cloned by three rounds of endpoint-dilution, 150 mls of *Lymantria dispar* cells grown in shaker flask were infected, budded virus was purified, viral DNA was isolated (Summers and Smith, 1987). Restriction site analysis was performed to confirm that correct recombination events had occurred and that the virus stocks were pure.

Assay of reporter gene expression at various times post infection

To examine the expression of the reporter genes during a time course of virus infection, *Lymantria dispar* cells (1.5 X 10^6) were seeded in 6-well tissue culture plates (Falcon) and allowed to attach for three hours. Cells were infected at an m.o.i. of 10 with the appropriate recombinant virus in 800 µl of medium for 1 hour. The medium was removed and replaced by 1 ml of fresh medium. For each timepoint, three independent wells were used. Cells were harvested at various time post infection with a rubber policeman, transferred to microfuge tubes and spun at 4000xg for three min. Supernatants were removed, cell pellets were resuspended in 500 µl of PBS (120 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.4)) and frozen at -80 ^{o}C. To assay for reporter gene activity, cells were lysed by one freeze-thaw cycle, debris was pelleted and 5 µl of supernatant was transferred to a 24 well plate (Falcon). To the 5 µl, 595 µl of 37 ^{o}C GUS assay buffer

(Jeffferson, 1987) was added quickly to all wells and incubated at 37 °C for 15 min. Three time points were taken every 15 min by removing 100 µl of the reaction mix and transferring it to a microfuge tube containing 900 µl of 0.2 M Na_2CO_3. The quanitity of the fluorogenic product from each time point was measured in a fluorimeter (Jefferson, 1987).

Western blots analysis

To verify that the recombinant viruses did not express the inactivated genes, Western blot analysis was performed. For analysis of p10 and PEP expression, *Lymantria dispar* cells infected with the recombinant viruses after 60 hrs.p.i. were lysed in 2X SDS-PAGE sample buffer and an amount of protein equivalent to approximately 4.8 X 10^4 cells was electrophoresed on each lane of a 10% SDS-polyacrylamide gel (Laemmli, 1970). Western blots were prepared as previously described (Quant-Russell et al., 1987). The antisera were diluted as follows: p39 (Mab 236) 1:7000; p10 (Mab 210) 1:5000; PEP 1:500 and p24 1:1000 in TBS-T (20 mM Tris (pH 7.5), 500 mM NaCl, 0.05% Tween 20) and incubated on Western blots for 2 hours. Blots were rinsed in three 10 min washes of TBS-T and incubated 1-2 hours in a 1:7500 dilution of goat anti-mouse IgG conjugated with alkaline phosphatase (Promega). Blots were rinsed (three times for 10 min) in TBS-T. Immunoreactive proteins were detected by incubating the blot in a substrate solution of 330 mg/ml nitro blue tetrazolium and 165mg/ml 5-bromo-4-chloro-3 indolyl phosphate.

Electron Microscopy

Insects were injected with BV wild type or recombinant OpMNPV at a 50,000 $TCID_{50}$. Fat body tissue was dissected out after incubation for 4 days and fixed in 2.5% glutaraldehyde, dehydrated with ethanol, and embedded in LR White resin as previously described (Russell and Rohrmann, 1990a). The p10 monoclonal antibody (Mab; 210) and the polyhedron envelope protein polyclonal antibody were used at dilutions of 1:1000 and 1:2000, respectively. Immunogold

labeling was carried out as previously described using 10 nm gold particles (Russell and Rohrmann, 1990a).

Results

Western blot analysis of wild type OpMNPV and recombinant viruses

To verify that the p10 and polyhedron envelope proteins were not expressed in the appropriate recombinant viruses, infected *Lymantria dispar* cell extracts from 60 hrs.p.i. were subjected to Western blot analysis. The p10-*lac* Z and p10-GUS recombinant viruses showed no expression of the p10 protein (Fig. 6.2a, lanes 1 and 2 respectively). The double recombinant virus (p10-*lac* Z and PEP-GUS) showed no expression of either p10 protein (Fig. 6.2a,lane 3) or polyhedron envelope protein (Fig. 6.2b, lane 1). The PEP-GUS recombinant virus showed no expression of the polyhedron envelope protein (Fig. 6.2b, lane 2). The wild type OpMNPV showed expression of both p10 protein (Fig 6.2a, lane 4) and polyhedron envelope protein (Fig 6.2b, lane 3). The p39-capsid monoclonal antibody (Mab 236) was used as a positive control for the analysis of p10 protein expression (Fig. 6.2a, lanes 1-4). The p24 polyclonal antiserum (see Chapter 2) was used as a positive control for the analysis of polyhedron envelope protein expression (Fig. 6.2b, lane 1-3).

Temporal expression of the p10 and PEP genes

To examine temporal expression of the p10 and PEP genes, *Lymantria dispar* cells were infected at an m.o.i. of 10 with the appropriate recombinant virus and were assayed for GUS activity various times post infection (see Methods). P10-GUS expression was initially observed at 20 hrs.p.i. and reached maximum expression at 96 hrs.p.i. (Fig. 6.3). In contrast, the PEP promoter GUS gene fusion activity from the double recombinant virus showed GUS expression beginning at 28 hrs.p.i. and reaching a maximum at 96 hrs.p.i. (Fig. 6.3). These results indicated that p10 expression began earlier and was approximately 12 fold greater than PEP expression at 96 hrs.p.i.

Orgyia pseudotsugata insect larvae infected with OpMNPV p10-*lac* Z and OpMNPV p10-GUS recombinants

Electron microscopy was used to examine the cytopathological and morpholgical effects caused by two recombinant virus which lacked the p10 gene and instead expressed *lac* Z or GUS reporter genes. No differences were evident between cells infected by these two virus. Therefore the data presented was from the recombinant virus with the p10 promoter-GUS gene fusion. No fibrillar structures were seen in the cytoplasm or in the nucleus, but high concentrations of what appeared to be microtubules were found in the same general locations (Fig. 6.4b). The lack of the p10 protein appeared not to have affected the shape of the polyhedra in infected insects (Fig. 6.4a). To determine the location of PEP in recombinants lacking p10, an anti PEP antiserum along with immunogold staining was employed. Intense immunogold staining was localized to the surfaces near the polyhedra (Fig. 6.4 a,c). This result indicated that p10 was not required for the transport or localization of PEP to the polyhedra surface. A distinct polyhedron envelope, however, was not observed in the recombinant (Fig. 6.4a,c) as compared to the wild type (Fig. 6.6c). However, on the polyhedron surface short patchy diffuse polyhedron envelope fragments were attached and stained heavily with immunogold specific for PEP (Fig. 6.4a). At higher magnifications, the polyhedra showed a diffuse zone that was different in texture from the internal part of the polyhedra and it stained with the PEP antiserum (Fig. 6.4c).

Orgyia pseudotsugata insect larvae infected with OpMNPV PEP deletion mutant

Insects infected with recombinant virus (PEP promoter-GUS gene fusion) that lacked a functional PEP gene, were examined by electron microscopy. The lack of PEP did not to affected the shape of the polyhedra (Fig. 6.5a). The distinct polyhedron envelope structure seen with the wild type (Fig. 6.6c) was never observed in the PEP-GUS recombinant virus (Fig. 6.5a). The fibrillar structures were observed (Fig. 6.5a,b) and immunogold labeling of the p10 showed staining

uniformly distributed throughout the fibrillar structures (Fig 6.5b). These results indicted that PEP was an essential component in the formation of the polyhedron envelope structure.

Orgyia pseudotsugata insect larvae infected with OpMNPV double recombinant p10-*lac* Z and PEP-GUS

Insects infected with the double recombinant virus were examined by electron microscopy for morphological changes. The most striking feature was that the cross sections of polyhedra show square or triangular profiles (Fig. 6.6a,b) as compared to wild type (Fig. 6.6c) This result indicated that double recombinant forms cubic polyhedra. Fibrillar structures and polyhedron envelopes were not observed in the double recombinant (Fig. 6.6a,b). Normal occlusion of the virions was observed (Fig. 6.6a,b). Neither the p10 or PEP minus virus showed a high frequency polyhedra with cubic morphology. These data suggested that the lack of p10 and PEP influenced the shape of the polyhedra.

Discussion

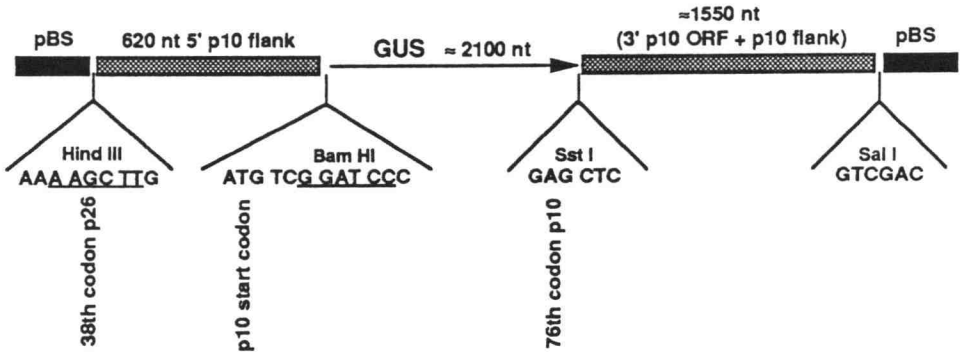
In this report, we have shown that both p10 and PEP were essential for the proper formation of the polyhedron envelope structure in OpMNPV. In *Autographa californica* MNPV, the 51 N-terminal amino acids of the p10 gene were fused to the β-galactosidase (*lac* Z) gene in a recombinant virus and examined in cell culture, and the resulting polyhedra did not display a polyhedron envelope structure (Williams et al., 1989). Another p10 minus recombinant virus was examined in which the 65 of the 94 amino acids were deleted from the C-terminal end of the p10 gene (Williams et al., 1989). This virus showed polyhedra with a polyhedron envelope that was patchy and not properly attached to the surface of the polyhedra (Williams et al., 1989). We have shown by immunogold labeling that the PEP was highly localized to the surface of the polyhedra in the recombinant viruses that contain a p10 promoter GUS or *lac* Z gene fusions. These results taken together would support the hypothesis that p10 plays a direct or indirect role in the proper formation or attachment of the polyhedron envelope structure rather than a transport function.

Orgyia pseudotsugata insect larvae infected with a OpMNPV virus lacking a functional PEP gene and of expression the GUS gene under the control of the PEP promoter showed no distinct polyhedron envelope structure. An AcMNPV recombinant that expressed the *lac* Z gene under the control of the PEP promoter also showed that polyhedra produced from this virus in cell culture did not have a polyhedron envelope (Zuidema et al., 1989; van Lent et al., 1990). An initial report on the composition of the polyhedron envelope showed that it consisted primarly of carbohydrates (Minion et al., 1979) but more recent reports have indicated that the polyhedron envelope also has a protein component (Gombart et al., 1989a,b; Whitt and Manning, 1988). The results presented here and those of others support the hypothesis that the PEP is an essential component in the formation of the polyhedron envelope structure.

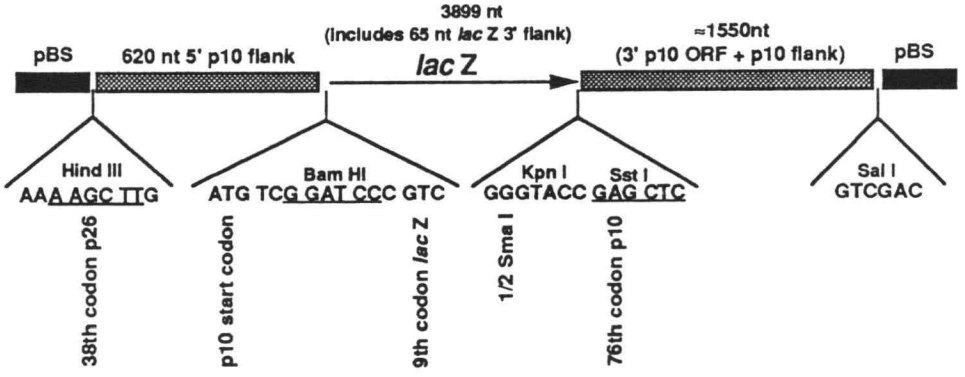
Polyhedra from *Orgyia pseudotsugata* insect larvae infected with a OpMNPV double recombinant virus which contained a p10 promoter *lac* Z and PEP promoter GUS gene fusions showed a striking cubic morphology. This result suggested that the cubic polyhedra morphology was caused by the lack of p10 and PEP. It is possible that PEP and p10 somehow block the formation of crystalline lattice such that cubic structures cannot form. However, there may be alternative explanantions for this phenotype. For example, a mutation in the polyhedrin gene that changes a leucine to a proline at amino acid 58 has been reported to cause a cubic morphology in the polyhedra (Carstens et al., 1986). We are currently replacing the *lac* Z gene with the p10 gene in the double recombinant. If the normal polyhedra morphology is restored then the possibility that a mutation had occurred in the polyhedrin gene or elsewhere in the genome will have been rule out. A less likely second explanation for the cubic morphology might be the co-expression of the two reporter genes in the infected insect cell. This possibility may be examined when a different double recombinant virus is constructed which expresses these two reporter genes.

Fig. 6.1 Maps of the transfer plasmids used for constructing the recombinant viruses. The construction of this plasmid is described in detail in the Methods. a) p10 promoter GUS gene fusion transfer vector. b) p10 promoter Lac Z gene fusion transfer vector. c) PEP promoter GUS gene fusion transfer vector. The solid bars represent the pBS sequences; the stippled and cross-hatched bars represent p10 and PEP flanking sequences respectively; the thin lines with the arrows at the end represent the Lac Z and GUS reporter genes. The nucleotide sequences of the cloning junctions are indicated.

a) p10-GUS transfer plasmid



b) p10-*lac* Z transfer plasmid



c) PEP-GUS transfer plasmid

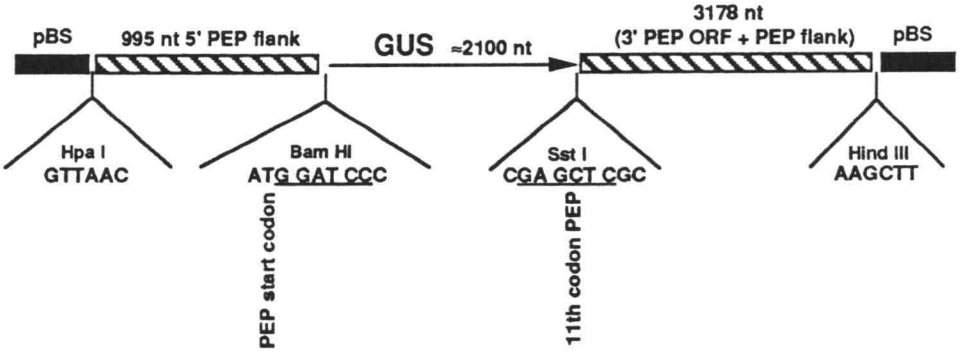


Fig. 6.1

Fig. 6.2 Western blot analysis of wild type OpMNPV and recombinant viruses. a) Western blot analysis using the p39 (Mab 236) and p10 (Mab 210) monoclonal antibodies. Total protein extracts from *Lymantria dispar* cells infected 60 hr.p.i. with the appropriate recombinant and wild type virus were blotted. The lanes contain: 1) p10-Lac Z recombinant virus 2) p10-GUS recombinant virus 3) p10-Lac Z and PEP-GUS double recombinant virus 4) wild type OpMNPV infected cell extracts. b) Western blot analysis using the PEP and p24 polyclonal antisera. The lanes contain: 1) p10-Lac Z and PEP-GUS double recombinant virus 2) PEP-GUS recombinant virus 3) wild type OpMNPV infected cell extracts.

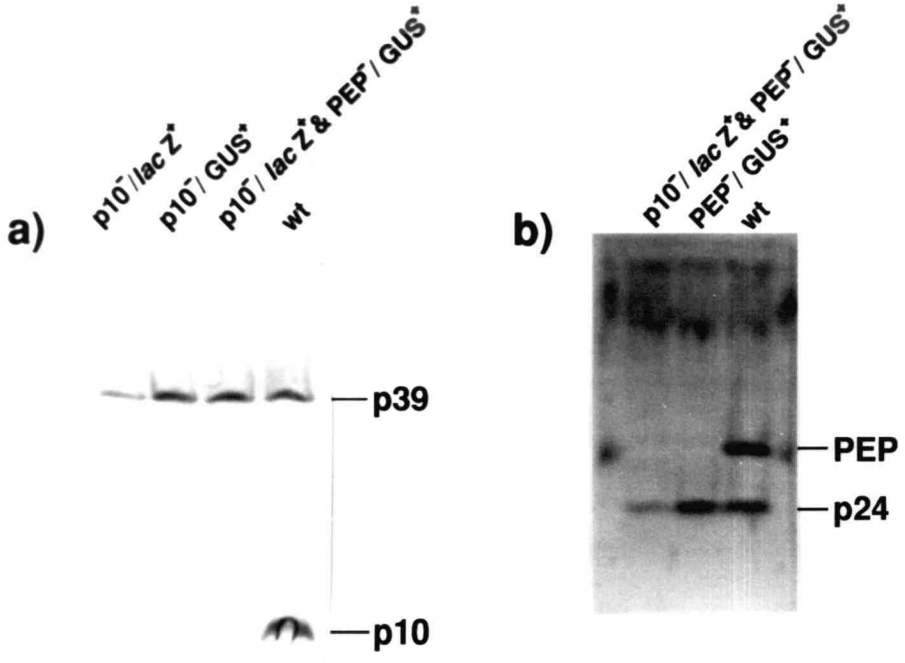

a)
p10⁻/lacZ⁺
p10⁻/GUS⁺
p10⁻/lacZ⁺& PEP⁻/GUS⁺
wt
p39
p10
b)
p10⁻/lacZ⁺& PEP⁻/GUS⁺
PEP⁻/GUS⁺
wt
PEP
p24

Fig. 6.2

Fig. 6.3 Temporal expression of three recombinant viruses that contained either a p10 promoter-GUS gene fusion or PEP-promoter GUS gene fusion.

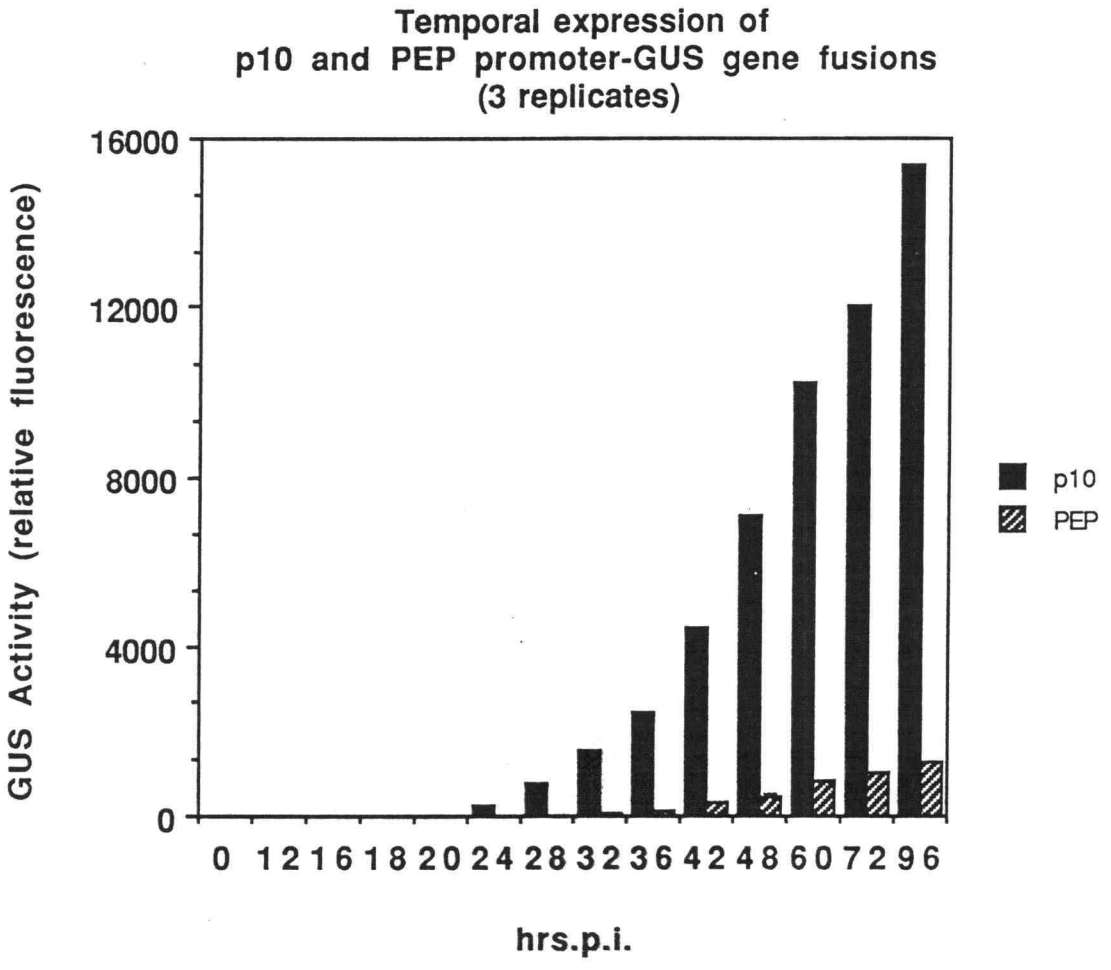

Temporal expression of
p10 and PEP promoter-GUS gene fusions
(3 replicates)
GUS Activity (relative fluorescence)
16000
12000
8000
4000
0
0 12 16 18 20 24 28 32 36 42 48 60 72 96
hrs.p.i.
p10
PEP

Fig. 6.3

Fig. 6.4 Electron microscopy of cells from *Orgyia pseudotsugata* larvae infected with OpMNPV p10 promoter-GUS gene fusion recombinant. a) Occlusion body immunogold stained with PEP antiserum. b) Microtubule-like structures c) Surface of an occlusion body at high magnification. The PEP antiserum was diluted 1:2000. The scale: 1 cm is equal to a) 0.2 μm, b) 0.5 μm, c) 0.1 μm.

a)

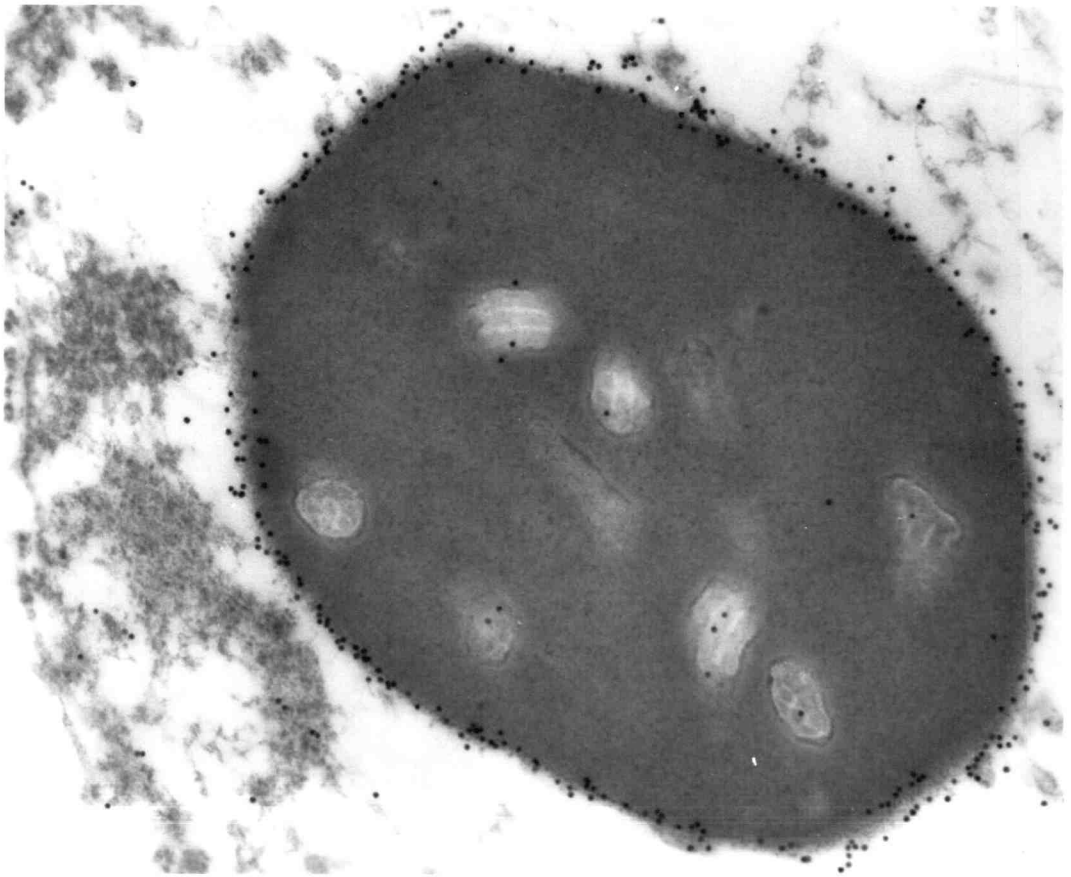
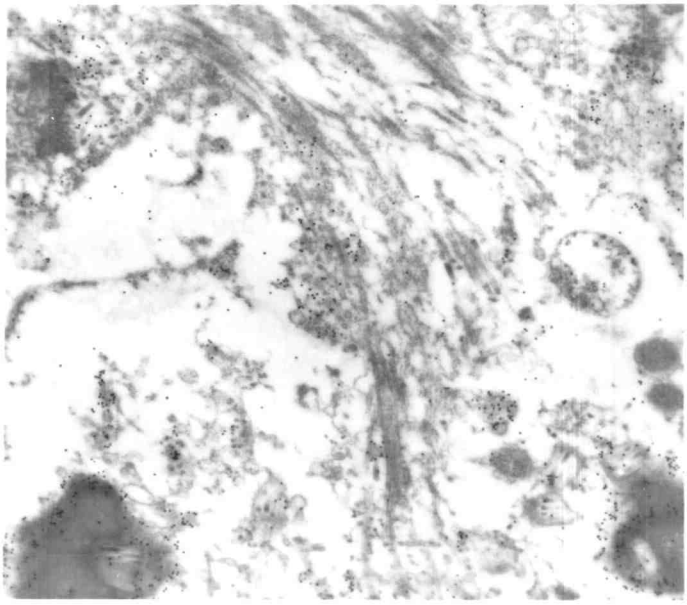


Fig. 6.4

b)



c)

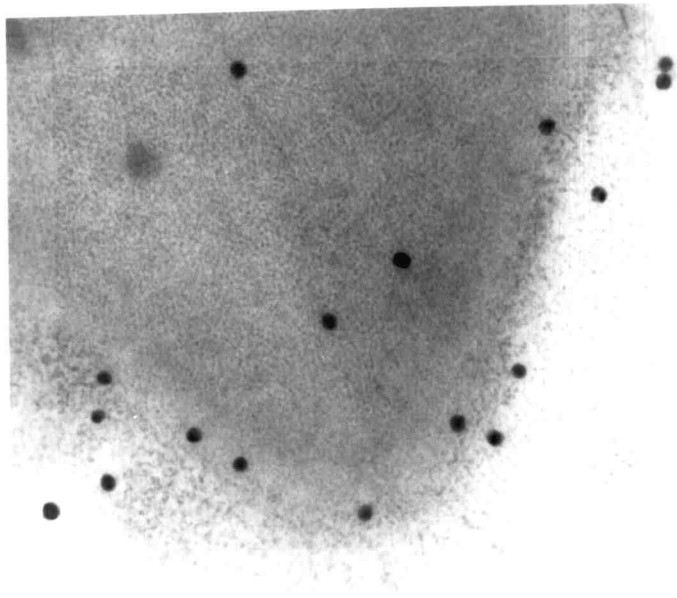


Fig. 6.4 continued

Fig. 6.5 Electron microscopy of cells from *Orgyia pseudotsugata* larvae infected with OpMNPV PEP-promoter GUS gene fusion recombinant. a) Occlusion body and fibrillar structure b) Fibrillar structure immungold stained with anti-p10. The p10 (Mab 210) antiserum was diluted 1:1000. The scale: 1 cm is equal to a) 0.2 µm, b) 0.2 µm.

a)

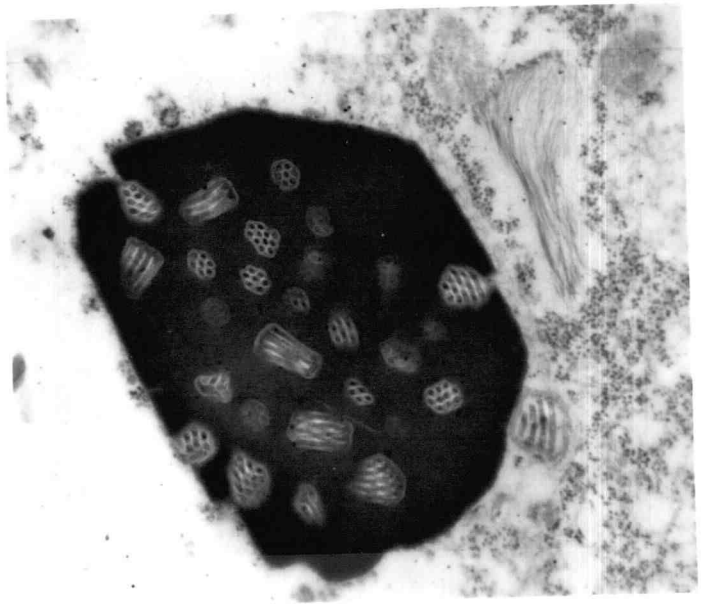


Fig. 6.5

b)

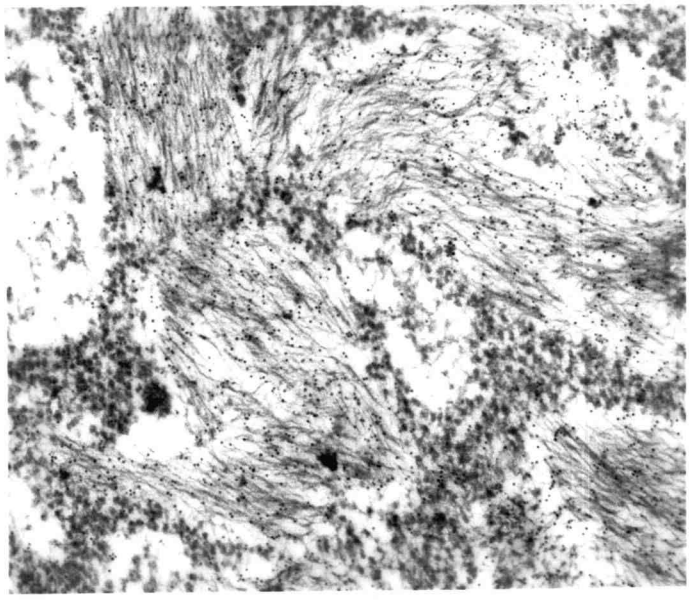


Fig. 6.5 continued

Fig. 6.6 Electron microscopy of cells from *Orgyia pseudotsugata* larvae infected with OpMNPV double recombinant p10 promoter-Lac Z gene fusion and PEP promoter-GUS gene fusion. a) infected fat bodies b) recombinant occlusion body c) wild type occlusion bodies. The scale: 1 cm is equal to a) 17.0 μm, b) 0.3 μm, c) 0.4 μm.

a)

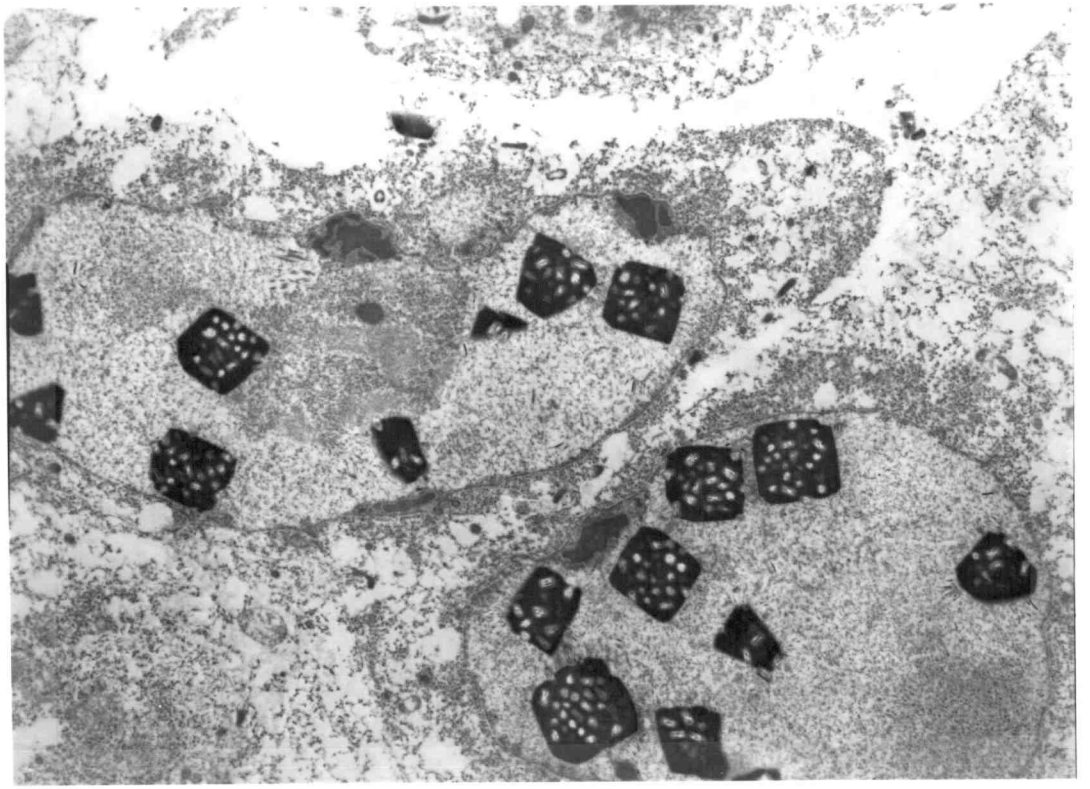
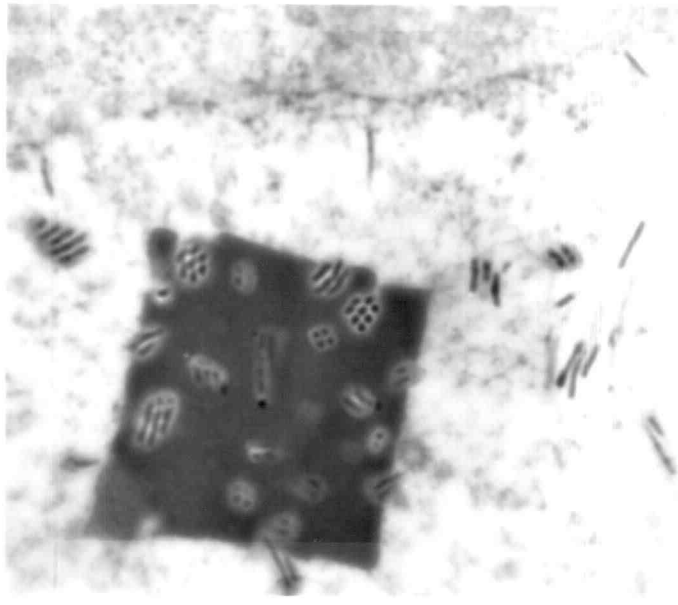


Fig. 6.6

b)



c)

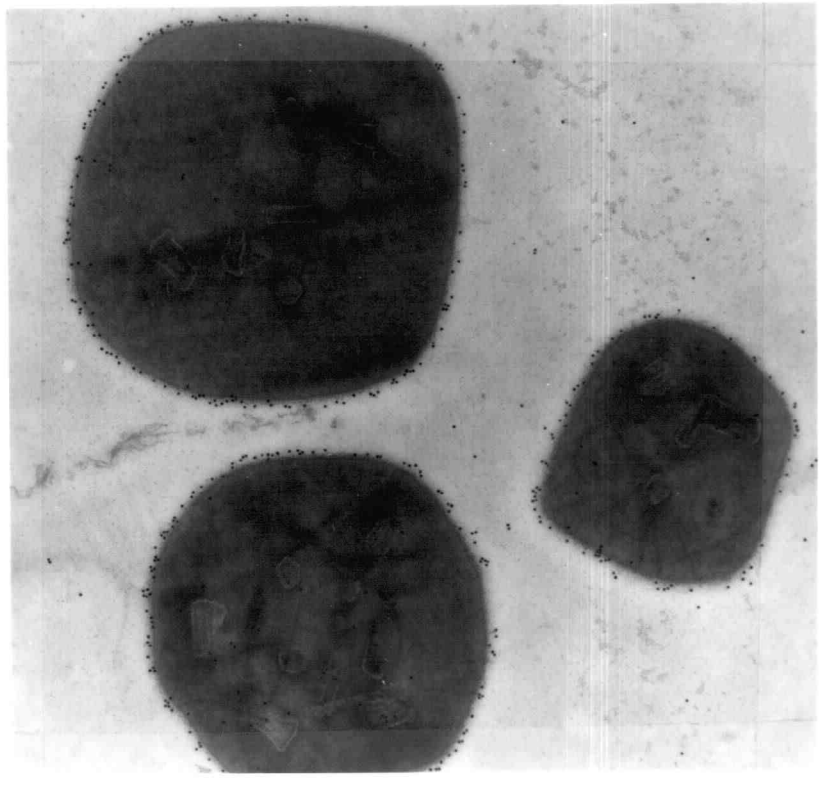


Fig. 6.6 continued

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APPENDICES

Appendix 1: Supplementary data for Chapter 2

Fig. A2.1 Relative CAT activity detected from the 3' restriction site deletion constructs. These restriction site deletions were initially constructed in attempt to localize the reduction in CAT activity. The 3' deletion constructs were constructed in the pPEPCAT plasmid using standard techniques. A restriction map of the polyhedron envelope protein gene region is shown at the top left of the diagram. The location and direction of ORFs 2-5 and the location of the CAT gene is shown directly below the restriction map. The restriction endonucleases used in the deletion constructs are indicated on the left. The number in parentheses is the deletion size in base pairs. Relative CAT activity is indicated on the X axis as counts per minute (above background) of ^{14}C-acetylated chloroamphenicol. Error bars represent the standard error detected from three independent transfections. The restriction site deletions had the following % CAT activity as compared to the parent plasmid: Bgl II/Sma I Δ 85%; Sst I/Sma I Δ 74%; Sma I/Apa I Δ 18%; Sal I Δ 5%; and Bgl II/Apa I Δ 4%.

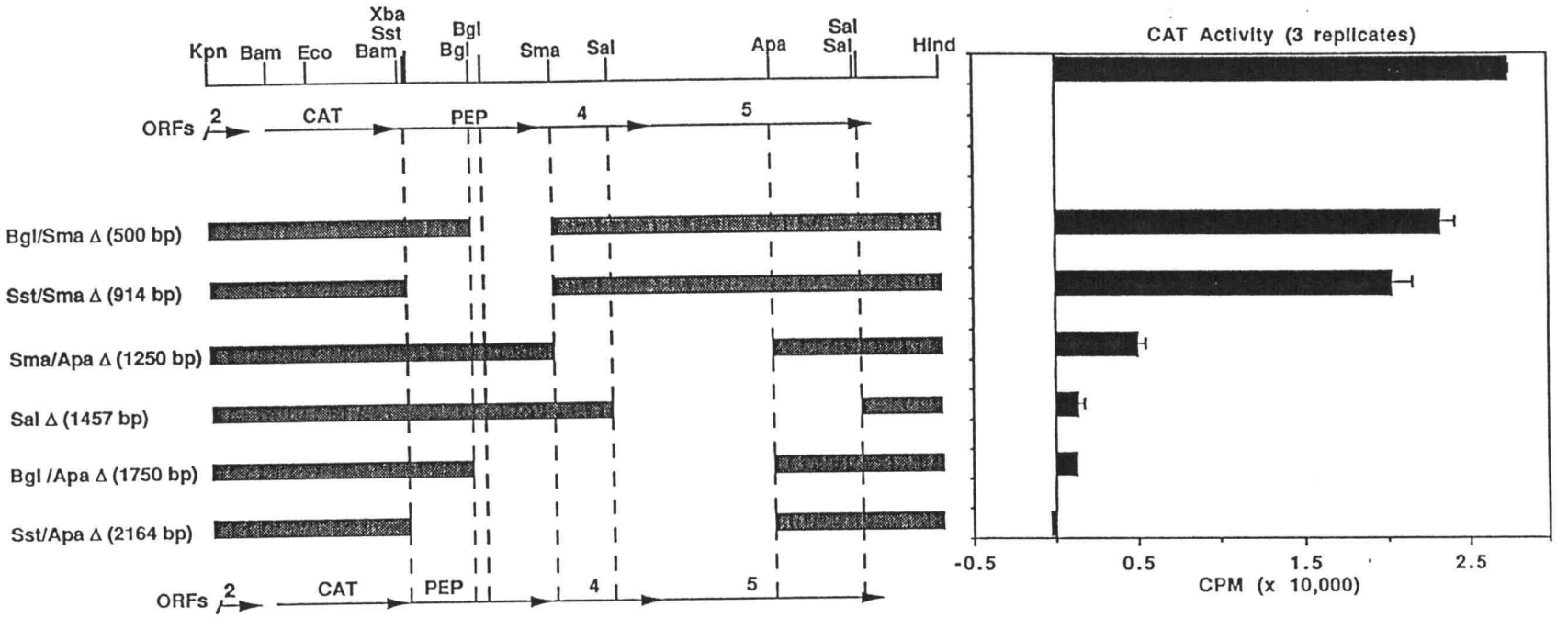

Kpn
Bam
Eco
Xba
Sst
Bam
Bgl
Bgl
Sma
Sal
Apa
Sal
Sal
Hind
ORFs
2
CAT
PEP
4
5
Bgl/Sma Δ (500 bp)
Sst/Sma Δ (914 bp)
Sma/Apa Δ (1250 bp)
Sal Δ (1457 bp)
Bgl /Apa Δ (1750 bp)
Sst/Apa Δ (2164 bp)
ORFs
2
CAT
PEP
4
5
CAT Activity (3 replicates)
-0.5
0.5
1.5
2.5
CPM (x 10,000)

Fig. A2.1

Fig. A2.2 Relative CAT activity detected from the 3' restriction site deletion constructs in the p1,2,PEPCAT plasmid. In this experiment I demonstrated that the reduction in CAT activity also occurred in the construct with all five ORFs present. A restriction map of the polyhedron envelope protein gene region is shown at the top of the diagram. The location and direction of ORFs 1-5 and the location of the CAT gene is shown directly below the restriction map. The restriction endonucleases used in the deletion constructs are indicated on the left. The number in parentheses is the deletion size in base pairs. Relative CAT activity is indicated on the X axis as counts per minute (above background) of ^{14}C-acetylated chloroamphenicol. Error bars represent the standard error detected from three independent transfections. The CAT activty of the Bgl II/Sma I Δ was 104% and the Sma I/Apa I Δ was 30% of the CAT activity of the parent plasmid.

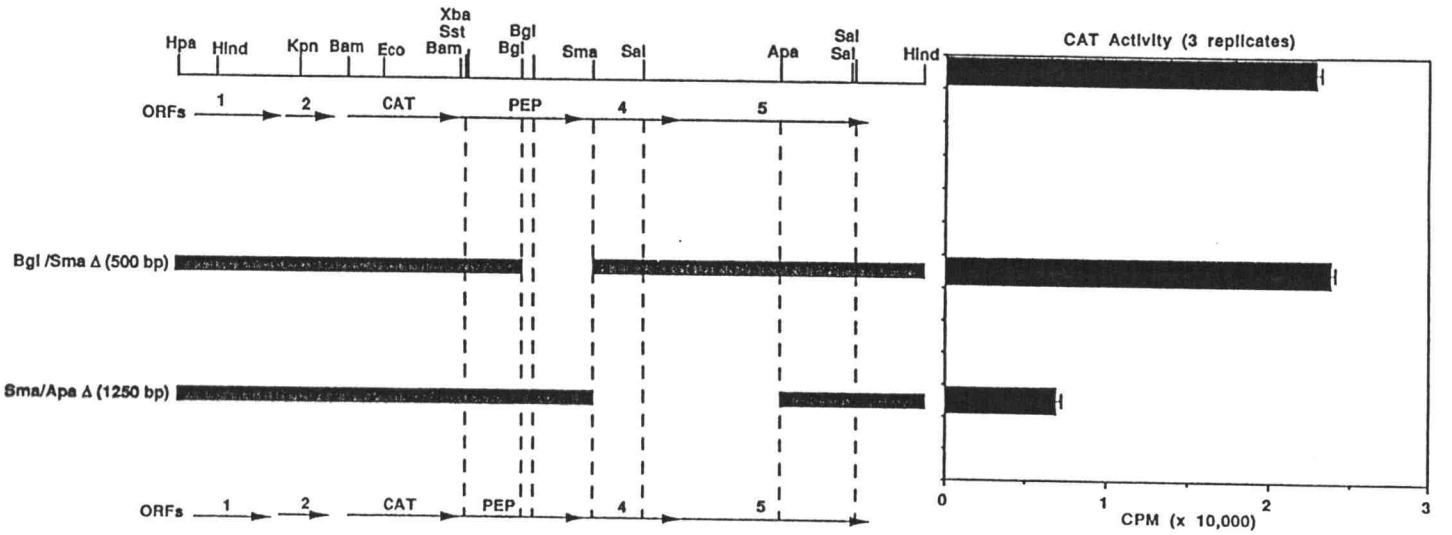

Hpa
Hind
Kpn
Bam
Eco
Xba
Sst
Bam
Bgl
Bgl
Sma
Sal
Apa
Sal
Sal
Hind
ORFs
1
2
CAT
PEP
4
5
Bgl /Sma Δ (500 bp)
Sma/Apa Δ (1250 bp)
ORFs
1
2
CAT
PEP
4
5
CAT Activity (3 replicates)
0
1
2
3
CPM (x 10,000)

Fig. A2.2

Fig. A2.3 Relative CAT activity detected from rescue constructs. The aim of this experiment was to differentiate between a length dependency or a sequence dependency in the 3' flanking sequence. To determine this the deletions were "repaired" by adding filler DNA that was comprosed of baculovirus DNA sequences lacking regulatory elements. The parent plasmid, deletion, and rescue constructs are indicated on the Y axis. Relative CAT activity is indicated on the X axis as counts per minute (above background) of ^{14}C-acetylated chloroamphenicol. Error bars represent the standard error detected from three independent transfections. The deletion size for the Sst I/Sma I D is 914 bp and had 82% of the parental CAT activity. The deletion size for the Sma I/Apa I D is 1250 bp and had 13% of the parental activity. The Sst I/Sma I deletion construct had a 750 bp fragment (Sau 3A fragment, Tomalski et al., 1988) of the AcMNPV DNA polymerase ORF inserted into the deleted region in both orientations. The result was no increase in CAT activity supporting a hypothesis that the 3' flanking sequence was sequence dependent.

The Sma I/Apa I deletion construct had a 1418 bp fragment (Bgl II to Sst I fragment, Müller et al., 1990) of the p87 ORF inserted into the deleted region in both orientations. The result was a 47% increase in CAT activity which would support a hypothesis that there was some length dependency. Alternatively, the p87 Bgl II to Sst I fragment may contain uncharacterized sequences that were capable of rescuing the 3' deletion constructs. The Sma I/Apa I deletion construct also had a 515 bp fragment (the first Sal I fragment, Blissard et al., 1989) of the p39 ORF inserted in the reverse orientation plus a 750 bp fragment of the DNA polymerase ORF inserted in the forward (or sense) orientation. This construct showed no increase in CAT activity which supports the sequence dependency hypothesis. These results indicated that the loss in CAT activity was influenced by sequence rather than the length of the 3' flanking sequence.

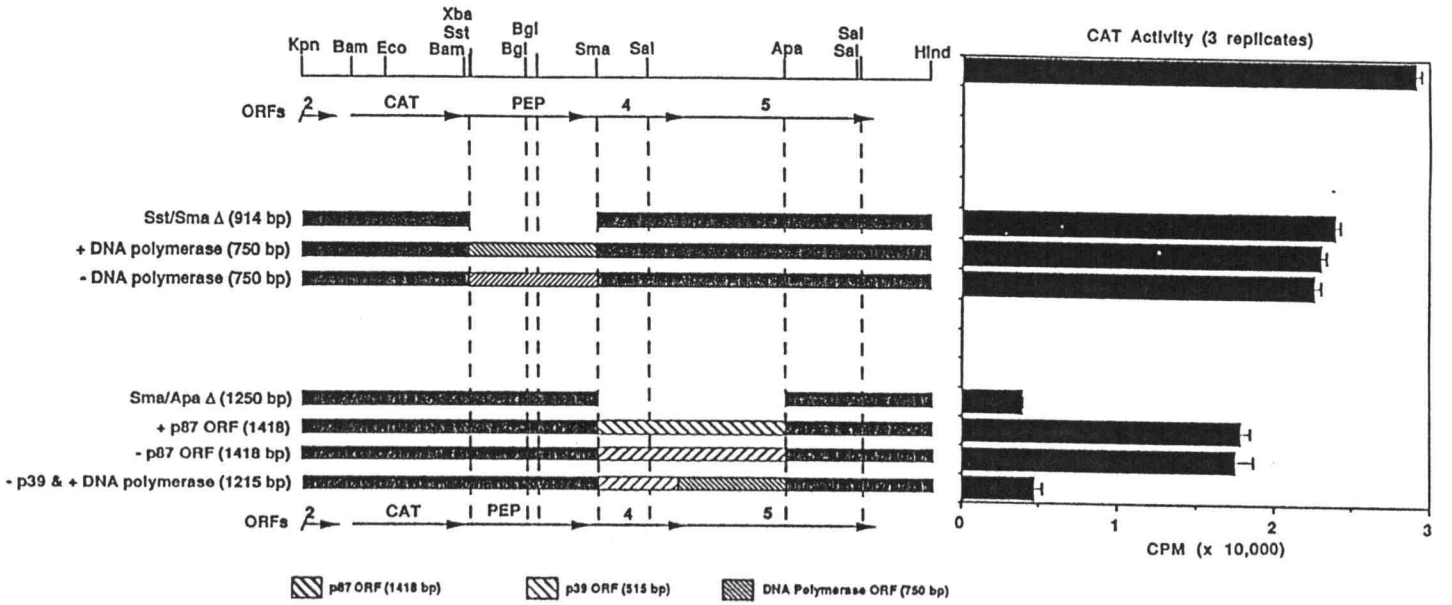

Kpn
Bam
Eco
Xba
Sst
Bam
Bgl
Bgl
Sma
Sal
Apa
Sal
Sal
Hind
ORFs
2
CAT
PEP
4
5
Sst/Sma Δ (914 bp)
+ DNA polymerase (750 bp)
- DNA polymerase (750 bp)
Sma/Apa Δ (1250 bp)
+ p87 ORF (1418)
- p87 ORF (1418 bp)
- p39 & + DNA polymerase (1215 bp)
ORFs
2
CAT
PEP
4
5
CAT Activity (3 replicates)
0
1
2
3
CPM (x 10,000)
p87 ORF (1418 bp)
p39 ORF (515 bp)
DNA Polymerase ORF (750 bp)

Fig. A2.3

Fig. A2.4 Relative CAT activity detected from constructs used to examine for the presence of an enhancer in the Sma I-Apa I fragment. The RNase protection analysis has shown that the decline in CAT activity was caused by a reduction in the CAT specific RNA levels. This result indicated that the effect was caused by either a transcriptional or a post-transcriptional mechanism. The aim of this experiment was to identify enhancer sequences in the 3' flanking sequences. The constructs were constructed in the pPEPCAT plasmid using standard techniques.

A restriction map of the polyhedron envelope protein gene region is shown at the top of the diagram. The location and direction of ORFs 2-5 and the location of the CAT gene is shown directly below the restriction map. The schematic representation of the constructs used to test for enhancer activity are indicated to the left. Relative CAT activity is indicated on the X axis as counts per minute (above background) of ^{14}C-acetylated chloroamphenicol. Error bars represent the standard error detected from three transfections. The CAT activity from each construct as compared to the parent plasmid were as follows: Sma I-Apa I deltion 20%; Sma I-Apa I fragment reversed 75%; Sma I-Apa I fragment 5' (upstream) and in the sense (forward) orientation of the transcription unit 72%; Sma I-Apa I fragment 5' (upstream) and in the anti-sense (reverse) orientation of the transcription unit 1%; An additional Sma I-Apa I fragment 5' (upstream) and in the sense (forward) orientation of the transcription unit 124%; and An additional Sma I-Apa I fragment 5' (upstream) and in the anti-sense (reverse) orientation of the transcription unit 105%. These results showed that there was not an enhancer sequence present. However, these data showed that there was orientation dependent and a position dependent activating sequence(s) present in the 3' flanking sequence.

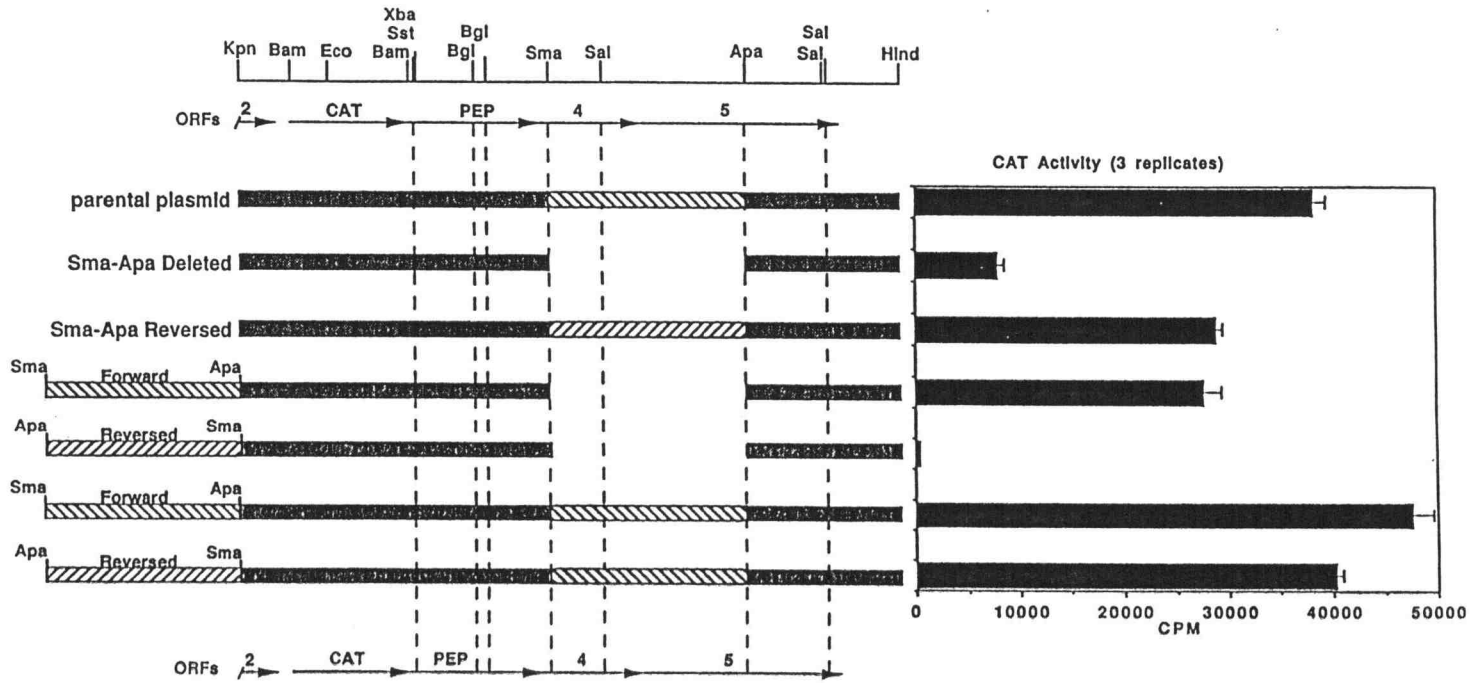

Xba
Sst
Bgl
Bgl
Sal
Sal
Kpn Bam Eco Bam Bgl Sma Sal Apa Sal Hind
ORFs 2 CAT PEP 4 5
CAT Activity (3 replicates)
parental plasmid
Sma-Apa Deleted
Sma-Apa Reversed
Sma Forward Apa
Apa Reversed Sma
Sma Forward Apa
Apa Reversed Sma
0 10000 20000 30000 40000 50000
CPM
ORFs 2 CAT PEP 4 5

Fig. A2.4

Appendix 2: DNA Mini-Prep Procedure

1. Transfer 1.5 ml of the 2.0 ml bacterial cultures into 1.5 ml eppendorf tubes. (Be sure your samples are numbered or labeled!)

2. Centrifuge the eppendorfs for 30-45 seconds. (You should now have bacteria pellets at the bottom of the tubes.)

3. Aspirate off the media supernatant, leaving the pellets on the bottom. Prepare a boiling water bath for step 7.

4. Add 300 µl STET to each tube. (To save on tips, squirt the liquid into the tube without touching the tip to the tube.)

	STET	100ml
sucrose	8%	8.0 g
Triton X	0.5%	0.5 ml
EDTA pH 8.0	50 mM	10.0 ml of 0.5M EDTA
Tris pH 8.0	10 mM	1.0 ml of 1M Tris

5. Prepare lysozyme cocktail: (Recipe shown is for 24 samples)

10 mg lysozyme
1.0 ml TE
(Mix by vortexing)

6. Add 25 µl of lysozyme cocktail to each eppendorf.

7. Vortex pellets into solution. Boil for 40 seconds in hot water bath.

8. Centrifuge for 7 min. Pull the white, stringy goobers out with a toothpick and discard.

9. Add 300 µl of isopropanol to each tube and mix. (Place another eppendorf rack over the top of the racked tubes and shake for 15 seconds.)

10. Centrifuge for 15 min. The DNA is now in tiny pellets at the bottom of the tubes. Carefully aspirate off the supernatant with a vacuum driven pastuer pipet that is capped with a sequencing pipet tip.

11. Add 100 µl of water to each tube, and vortex briefly. Resuspension is much easier if allowed to soak a half hour to an hour. This is a good place to stop by storing the tubes at 4°C for less than a week or -20°C for greater than a week. If the plasmid DNA is to be used for sequencing, it should be stored at 4°C only, and used within a week.

12. Resuspend pellet with a yellow pipet tip or by vortexing.

13. Transfer 5 µl to a 0.5 ml eppendorf. (Be sure to label these, too!) If your DNA is a pATH vector, use 10 µl because it has a lower copy number. Accordingly, you should reduce the water in step 14 by 5 µl.

14. Mix the restriction enzyme cocktail. Make 15 µl of cocktail for each tube, plus a little extra. Recipes shown are for one sample.

	one restriction enzyme	two enzymes
enzyme #1	0.3 µl (10 units/µl)	0.3 µl
enzyme #2	none	0.3 µl
10X buffer (REACT)	2.0 µl	2.0 µl
distilled water	12.7 µl	12.4 µl
	15.0 µl	15.0 µl

15. Add 15 µl of restriction enzyme cocktail to each tube containing DNA, mix (by pipeting up and down) and incubate in 37 °C water bath for 1 hour.

16. Add loading buffer in order to run the samples on a gel. If you expect DNA fragments of less than 1000 bp, then you need to have RNAse A in your loading buffer so you can see the smaller bands on the gel. (Consult Sambrook et al., 1989 for preparation of RNAse A.) Recipes for one tube:

	DNA > 1000 bp	DNA < 1000 bp
loading buffer	5.0 µl	4.5 µl
RNAse A (10 mg/ml)	none	0.5 µl

17. Gels can be made in TAE or TBE buffer. TAE should be used if bands of DNA are to be recovered. TBE should be used if high resolution of the DNA bands is important. TBE also allows gels to be run faster, because higher voltage may be used.

50x TAE (2L)	10x TBE (2L)
484.0 g Tris	216.0 g Tris
74.4 g EDTA	7.44 g EDTA
114.0 ml Acetic acid	110.0 g Boric acid

The amount of agarose in the gel depends on the fragment size of the DNA:

DNA fragment size	% agarose
100 bp to 3 Kb	2.0%
200 bp to 4 Kb	1.5%
500 bp to 6 Kb	0.9%
> 6 Kb	0.6%

For example, for a 0.9% TAE gel, use 100 ml 1X TAE and 0.9 grams of agarose. Add 0.5 µl of ethidium bromide per 100 ml of 1X TAE.

18. Load each tube containing 25 µl of sample into a well of the gel. Do not forget to load the one Kilobase ladder as size standard.

Appendix 3: Vector Cipping Procedure

Component	volume for 10 µg of DNA	volume for 5µg of DNA
DNA in restriction buffer	100 µl	50 µl
1 M Tris pH 8.0	5 µl	2.5 µl
1:10 Diluted cip enzyme in TE pH 8 (undiluted cip is at 1 unit/ml)	10 µl	5 µl

Incubate @ 37°C for 15 min then
@ 55°C for 15 min

If 3' recessed or blunt ends then add another half aliquot of diluted cip.

Incubate @ 37°C for 15 min then
@ 55°C for 15 min

Immediately Phenol / Chloroform extract
then
Do one of following:

1) Add:
1 volume of 7.5 M NH_4Ac
2 volumes of 95% ETOH

Store @ -80 °C for 15 min
Spin @ 4 °C for 15 min
Remove Supernatant
Respin @ 4 °C for 5 min
Remove remaining supernatant
Resuspend in TE
Vector may now be used for cloning

2) Run out on TAE gel.

Appendix 4: Large Scale Plasmid Isolation

1) Pellet 250 mls of bacteria in Sorvall at 5K for 20 minutes

2) Gently resuspend bacteria in 5 mls
   25 mM Tris HCl pH 8.0
   10 mM EDTA
   50 mM Glucose

3) Transfer cell suspension to a sterile 125 ml flask

4) Add 10 mg (2 mg/ml) of lysozyme directly as powder and mix well

5) Incubate on ice for 30 mins

6) Add 5 mls of Alkaline-SDS solution

Final conc.	
1% SDS	250 ml of 20% SDS
0.2 M NaOH	200 ml of 5 M NaOH
	added to 4.55 mls MQ water

7) Mix throughly, incubate on ice for 5 mins

8) Add 3.75 mls of 3 M Na Acetate pH 4.8, Mix throughly

9) Incubate on ice for 30 mins mixing every 15 mins

10) Transfer the solution to a sterile 30 ml Corex tube

11) Centrifuge at 10,000 rpm for 15 mins at 4°C

12) Remove as much (if any) loose white floculant material from the top of the tube as possible with a sterile toothpick. Pour the supernatant into a new sterile 30 ml Corex tube.

13) Add equal volume of isopropanol mix and leave at -70°C for 15 mins

14) Centrifuge at 10,000 rpm for 15 mins at 4°C

15) Pour off the supernatant and leave the tube upside down for a few minutes to allow the isopropanol to drain off

16) Resuspend the pellet in 3 mls of TE pH 8.0

17) Add 1.5 mls of 7.5 M Ammonium Acetate, mix and leave on ice for 20 mins

18) Centrifuge for 15 mins at 10,000 rpm at 4°C **Save Supernatant**

19) Pour the supernatant into a new sterile Corex tube and add two volumes of 95% Ethanol

20) Leave at -70°C for 15 mins

21) Centrifuge for 20 mins at 10,000 rpm at 4°C

22) Drain the pellet well,

If a CsCl gradient is to be run (using a VTi 80 rotor) then resuspend the pellet in 4.22 mls of TE pH 8.0 Transfer to a sterile 15 ml conical centrifuge tube and add exactly 4.33 grams of CsCl. Mix throughly until completely dissolved and then add 75 ml of ETBr (10 mg/ml) Transfer solution to an ultra centrifuge tube.

After the Cscl, run transfer the DNA solution into 1.5 ml eppendorfs. Extract three times with an equal volume of butanol saturated with 10 mM Tris pH 8, 1 mM EDTA, 5 M NaCl to remove the ETBR.

Centrifuge briefly after each extraction to set phases. Remove the pink top layer to organic waste bottle.

After the last extraction, combine the 1.5 ml eppendorfs that contain the same sample into a 15 or 30 ml corex tube. Add equal volume of MQ water, mix, and then 2 volumes of 95% ETOH. If there was a lot of DNA appears, centrifugation may take place right away. Otherwise leave at 4 ^{o}C overnight. Be sure the CsCl does not come out of solution. If CsCl comes out of solution, warm tube to room temperature until solubilization is complete.

If no gradient is to be run then proceed to step 23.

23) Resuspend the pellet in 2 mls of TE pH 8.0 and transfer to a sterile 15 ml Corex tube

24) Add RNase to a final concentration of 0.5 mg/ml from 10 mg/ml stock that has been treated so that RNase solution is DNase FREE (Add 100 ml of 10 mg/ml RNase stock to 2 mls)

25) Incubate on ice for 30 mins

26) **Phenol/Chloroform extract:** Add 1/2 volume of phenol vortex and add 1/2 volume of chloroform and vortex

Centrifuge at 10,000 rpm at 4^{o}C for 15 mins

Transfer supernatant to a new sterile 15 ml Corex tube and add an equal volume of Chloroform and vortex

Centrifuge at 10,000 rpm at 4^{o}C for 15 mins

Transfer supernatant to a new sterile 15 ml Corex tube. Add equal volume of 7.5 M Ammonium Acetate (2 mls) and then two volumes of 95 % Ethanol (8 mls)

27) Leave at -70^{o}C for 15 mins

28) Centrifuge at 10,000 rpm at 4^{o}C

29) Resuspend pellet in 1 ml of TE pH 8.0 and transfer to a 1.5 ml eppendorf tube.

Appendix 5: Viral DNA Isolation Procedure

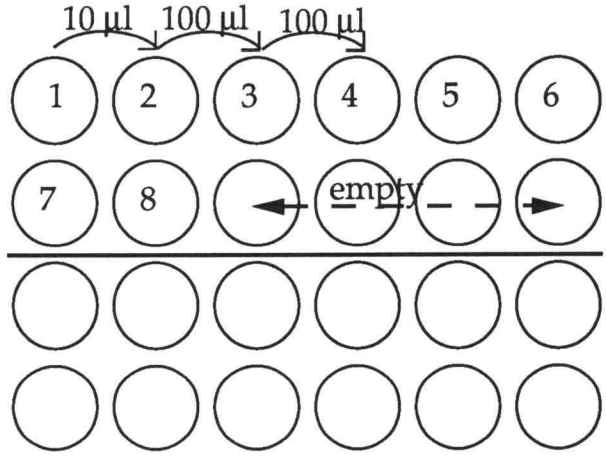
1) Spin out the insect cells in the International harvester at 2500 rpm for 15 mins
2) Save Supernatant (may be stored at 4 °C until ready to use) and place in SW28 tube (fill up to the top with TE pH 8) and balance
3) Spin in the ultracentrifuge at 24,000 rpm for 30 min at 4 °C
4) Drain pellet and bring up in 500 ml of extraction buffer (Summers Manual page 24). Transfer to 1.5 ml eppendorf

For 1 liter of extraction buffer:
12.1 g Tris pH 7.5
33.6 g $Na_2EDTA\ 2H_2O$
14.9 g KCl

5) Add 2 µl of 10 mg/ml of proteinase K (20 mg)
6) Incubate at 53 °C for 2 hours
7) Add 50 µl of 10% Sarkosyl and incubate at 53 °C overnight
8) Be careful not to shear DNA from here on out (use sterile 5 ml pipet to transfer liquid)
9) Phenol/Chloroform extract twice
10) Ethanol precipitate add two volumes of ethanol (Add No Salt) Leave at -80 °C for 20 mins or -20 °C for 2 hours or longer
11) Pellet DNA at low speed (2,500xg for 30 mins)
12) Resuspend DNA in 250 µl of TE pH 8 and incubate at 65 °C for 15 mins. Then overnight at 4 °C
13) Sometimes a lot of RNA is also isolated. It therefore becomes necessary to quantify the amount of DNA on a gel before setting up a digest. (Normally requires about 50-100 µl of DNA for a digest)
14) The DNA is usually digested in 100 µl reaction and then ethanol precipitated and brought up in a smaller volume to load onto a gel.

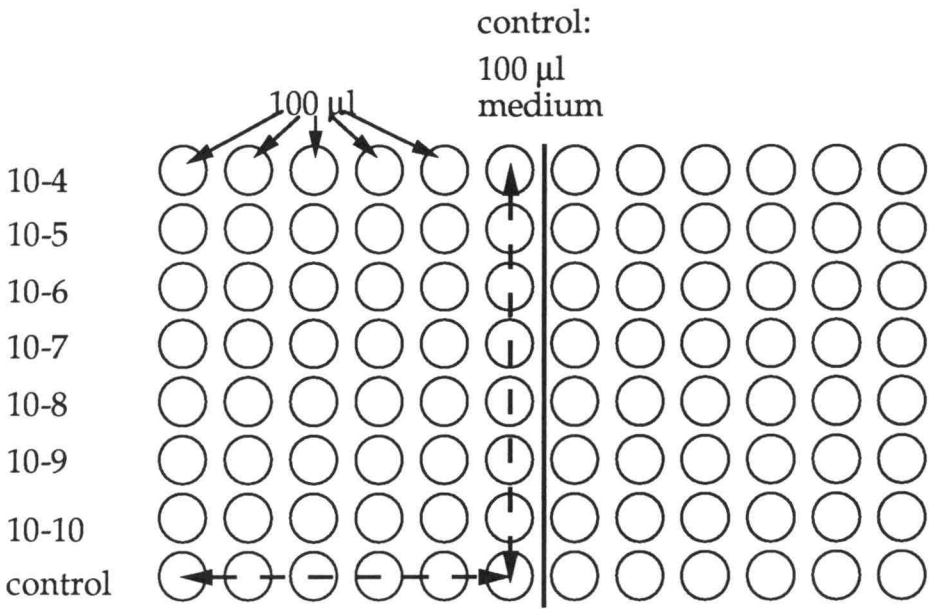
Appendix 6: Virus Titration Procedure

1. Figure cell concentration
2. Dilute cells to 5E5 cells/ml
3. In a 96 well plate, place 100 µl of cells so that there are 5E4 cells per well. Half the number of cells should be used when cytopathology will be used to determine infection. Use a multipipetor for this task. Incubate at 27 C overnight so that cells attach to plate. However, cell attachment is not necssary.
4. For the viral dilutions, you need 8 wells of a 24 well plate for each virus. In wells 1 and 2, put 990 µl of medium. Put 900 µl of medium in the other wells.
5. Put 10 µl of virus in well 1 and mix. With a new pipet tip, transfer 10 µl to well 2 and mix. Transfer 100 µl from well 2 to well 3 with a new tip and mix. Then 100 µl from 3 to 4, and so forth.



Well	Dilution:
1	10-2
2	10-4
3	10-5
4	10-6
5	10-7
6	10-8
7	10-9
8	10-10

6. Add 100 µl of the titered virus to each 96 well according to the diagram on the next page. The wells covered by dashed arrows are control wells and 100 µl of medium, not virus, should be added to these.

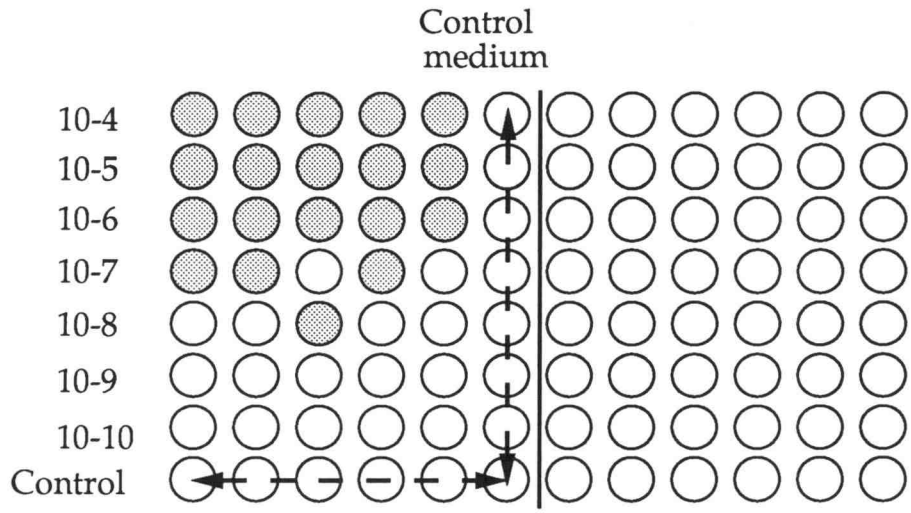


7. Put the sticky plastic film over the wells to reduce evaporation. You should also put the plates in a plastic bag and include a damp paper towel to maintain a high level of humidity.
8. Incubate for 5 days at 27 C.
9. For a more accurate endpoint, it is necessary to amplify the virus. This is done by making a copy of the plate that was set up in step 6. To do this, put 100 µl of cells in each well of a 96 well plate, as in step 3. Then transfer 100 µl of virus from the step 6 plate to the corresponding wells in the new plate. Use a multipipet for this task. Incubate for 5 days and then check to see which wells contain a virus. However, when using a reporter gene as an identifier amplification is not necessary.

Calculations:

1. Look at the amplification plate. Fill out the table below, including a column for the last totally positive dilution, the first totally negative dilution, and every dilution between. For example, if your amplification plate looks like this:

Amplification plate from step 9:



The table would look like this, with four columns. Definitions of the rows given below the table.

Dilution	10-6	10-7	10-8	10-9
positive rate	5/5	3/5	1/5	0/5
positive number	5	3	1	0
negative number	0	2	4	5
positive total	9	4	1	0
negative total	0	2	6	11
pos. ratio total	9/9	4/6	1/7	0/11
percent positive	100	67	14	0

positive rate: the ratio of positive wells to the total wells for each dilution; the number of wells in this protocol is 5 per dilution.
positive number: the number of positive wells for each dilution; this is the numerator of the positive rate.
negative number: the number of negative wells for each dilution; this is equal to 5 - positive number.
positive total: the sum of the positive wells for the given dilutions and all higher dilutions. It is the summation of the positive number of the given column and each positive number to the right. Inspect the sample to alleviate confusion.
negative total: the sum of the negative wells of the given dilution and all lower dilutions; the opposite of the positive total. It is the summation of the negative number for each dilution and all negative numbers to the left in the table. Again, inspect the sample.

***positive ratio total*:** Use the following equation:

positive ratio = positive total
total positive total + negative total

percent positive: this is simply the positive ratio total converted to a percent.

We can now use the above table to figure out the 50% endpoint:

1. Take the log of the lowest dilution that does not have all positive wells (next above 50%). Example: log 10-7= -7

2. Figure out the proportional distance with the following equation:

proportional distance =

(% pos. next above 50%)-50%
(% pos. next above 50%)-(% pos. next below 50%)

67%-50% = 0.32
67%-14%

3. Subtract the proportional distance from the log of the lower dilution.
-7 - 0.32 = -7.32

4. The tissue culture infectious dose (TCID) for 50% infection is ten raised to the power of your answer for step three:

$10^{-7.32} = 4.8E\text{-}8$

5. To convert to milliliters, take the reciprocal of your answer for step 4 and divide by .01, since one one-hundredth of a milliliter was originally taken in step 5 of the procedure.

4.8E-8 = 1/ 2.1E7

2.1E7/.01 = 2.1E9 TCID(50)/ml

6. Assuming that 50% of the virions form plaques (a 50% infection efficiency), multiply by 0.5 to find the number of plaque forming units (PFU) per milliliter.

2.1E9 TCID/ml x 0.5 = 1.05E9 PFU/ml

Appendix 7: Mini-Prep dsDNA Sequencing with Sequenase

1) Use 2-4 µg of mini-prep DNA for plasmid sequencing.

a. A normal 1 ml mini-prep will yield 5-7 µg of DNA. Therefore, use ≈ 40%.

b. Mini-preps should be processed by the boiling method, precipitated in isopropanol, and resuspended in 100 µl of MQ H_2O.

c. The dsDNA from the miniprep should be used as is without RNAse treatment.

2) Add 10 ng of primer

The primer : template should be a 10:1 ratio for best results.

3) Add 2 M NaOH, 2 mM EDTA and denature.

Denature the dsDNA/primer mixture at 85 °C for 5 mins. Chill on ice for 3 mins.

EXAMPLE:

40 µl of mini-prep DNA (from 100 ml Mini preps)
4 µl of Primer (@2.5 ng/µl)
5 µl of 2 N NaOH, 2 mM EDTA
49 µl

4) Neutralize the mixture. Ethanol precipitate.

Add: 6 µl of 3 M NaOAc (5.2), mix and add 150 µl of EtOH

Precipitate @ -80 °C, 10 minutes.

5) Pellet DNA in 4 °C microfuge, 15 minutes.

6) Resuspend DNA pellet in 8 µl of H_2O

7) Add 2 µl of 5X Sequenase Buffer and mix.

8) Anneal @ 37 °C for 15 minutes.

9) Dilute dITP Labeling Mix 1:3 in H_2O

For cloning site sequencing dilute labeling mix 1:5 or 1:10. Also use a 12% gel.

10) Dilute Sequenase Enzyme 1:8 in ice-cold TE buffer [10 mM Tris (7.4), 1 mM EDTA]

Store on ice.

Note: Use diluted enzyme within 1 hour after diluting.

11) LABELING REACTION:

To the tube containing the annealed template/primer, add:

1.0 µl of 0.1 M DTT
2.0 µl of Labeling Mix (dITP mix, Diluted 1:3)
0.5 µl of ^{35}S-alpha-dATP (10 µCi/µl, 1000-3000 Ci/mmol)
2.0 µl of Sequenase Enzyme (freshly diluted)

Incubate @ RT for 1-10 minutes.

Note: To get more label in the smaller fragments:
Dilute the Labeling mix 1:10 or 1:15
Incubate for shorter periods 2-3 min.

To sequence long a distance:
Use the Labeling Mix undiluted
Add 1.5 µl ^{35}S-dATP (15 µCi)
Incubate @ RT for 10 minutes

12) TERMINATION REACTION:
Add 3.5 µl of from the above tube to all 4 termination tubes, each containing 2.5 µl of the appropriate dideoxy termination mix (dITP; G, A, T, or C)

Incubate @ 37 ^{o}C for 15 to 30 minutes

13) Add 4 µl STOP Buffer to each tube

Store @ -20 °C.
Load 1µl per well (96 well comb) or 2 µl per 48 well comb

8% INSTA-GEL:
38 g Acrylamide
2 g Bis-acrylamide
210 g Ultra Pure Urea
50 ml 10X TBE running buffer
MQ Water to 500 ml of final volume

Filter through Whatman #1 filter paper
Store @ RT in a foil wrapped bottle.
[Note: insta-gel will go bad after approximately 6 months _ it will polymerize very fast but is still be usable. If old, use only about 1/2 the amount of 10% ammonium persulfate as per normal and pour fast]

To 70 ml of Insta-Gel, add 0.6 ml of freshly made 10% Ammonium persulfate in MQ H_2O and mix

Add 15 µl of TEMED, mix and immediately pour the gel.
Allow the gel to polymerize for 35 min to 1 hr (be sure it is completely polymerized)

Component	6 %	7 %	8 %	9 %	10 %
40 % acrylamide (1:19 ratio)	11.2 ml	13.1ml	15.0ml	16.9ml	18.8ml
10x TBE	7.5 ml				
Urea	34.5 g				
Water					
Total	75 ml				

Appendix 8: Cell Culture Transfection Procedure

STEP 1

1) Prepare cells for transfection by removing the old medium and adding 10 mls of fresh medium to a confluent T-150 flask (confluent T-150 contains roughly 2.5 X 10^7 viable cells).

2) Knock off cells and transfer to a 50 ml Falcon tube.

3) Take 0.5 ml into 13 X 100 mm culture tube and add 50 µl of 0.5% Trypan Blue in 2 X PBS (use 1: 10).

4) Let sit for 2 mins.

5) Prepare and load the hemocytometer. Remember to swirl the tube just before loading because the cells settle out quickly.

6) Take the actual number counted in the field and use the following formula.

Actual # X 10^4 = cells/ml

7) Place 1.5 X 10^6 cells in each well of a six well plate (Falcon #3046).

8) Allow cells to attach for about 4 hours to overnight.

9) Determine the correct amount of DNA and mix with 400 µl of transfection buffer. For example : If 20 µg of DNA is to be transfected mix 25 µg with 500 µl of transfection buffer. The extra 100 µl and 5 µg of DNA allows for pipeting error. Allow the mixture to come to room temperature before using.

STEP 2

1) Remove the medium from each well and replace it with 800 µl of medium.

2) Add the correct MOI of virus to each well. (The virus may be premixed with 800 µl of medium and then dispensed) **Always do the low multiplicity of infection first.**

3) Incubate the plates for 4 hours.

4) Remove the 800 µl of media containing the virus.

5) Add 400 µl of Graces with 10% Fetal Bovine Serum and Antibiotics to each well.

6) Rock the plate a couple of times to allow the liquid to spread evenly throughout the well.

7) Now add 400 µl of transfection buffer containing the DNA. **Add in a dropwise fashion evenly throughout the well.**

8) Rock the plate a couple of times to allow the liquid to spread evenly throughout the well.

9) After 4 hours take off the liquid and add 1 ml of media.

10) Put in a zip lock bag with a wet paper towel and incubate for 72 hours.

STEP 3

1) To harvest the transfected cells scrape the cells loose with a sterile rubber policeman.

2) Transfer to a 1.5 ml eppendorf tube.

3) Spin at 60% for 3 mins.

4) Take off supernatant; spin again for 15-20 sec at 60%.

5) Take off supernatant.

6) Add 50 µl of 1 X PBS + 5 mM EDTA (Filter sterilized).

7) Vortex and freeze at -80 °C for 5 mins (can stop at this point).

8) Thaw at room temperature for 5 mins

9) Vortex

10) Repeat steps 7-9 three times.

11) To destroy de-acetylating activity, heat the extract to 65 °C for 15 mins.

12) Pellet the cell debris (10 mins in microfuge) and transfer the supernatants to new tubes.

13) Store the supernatant extract at -80 °C

14) Determine the total protein concentration by using Bradford Assay

Solutions

Transfection Buffer	**For 1 liter**
25 mM Hepes (238.3 g/mole) sigma H-3375	5.96g
140 mM NaCl (58.44 g/mole)	8.18g
125 mM $CaCl_2$ 2 H_2O (Fw 147.03)	18.38g

Filter Sterilized pH 7.1 at room temperature (25 °C)

1X PBS-EDTA

495 ml of 1x PBS Sigma premix (pH 7.4) and 5 ml of 500 mM EDTA (pH 7.1)

Appendix 9: CAT Assay Procedure

Two Phase Fluor Diffusion Assay from NEN

1) Infect or transfect insect cells with a construct that has a Promoter-CAT gene fusion.

For transfections, I typically use: 1.5 x 10^6 cells/well in a 6 well plate (35 mm diameter wells) and transfect with 10 µg of plasmid DNA in a total volume of 0.8 ml (0.4 ml Graces complete + 0.4 ml transfection buffer w/ DNA)
After 4 hrs, the transfection buffer is removed and replaced with 1 ml of TNM-FH. Hours post transfection (hpxf) is calculated from the time when the 1 ml of TNM-FH is added. See Appendix 8

2) At 72 hpxf, scrape the cells off with a rubber policeman and pipet the cells/media into an eppendorf tube.
Pellet the cells gently in the microfuge 3 min @ 60% setting (Dupont microfuge). Carefully remove and discard the supernatant. Respin briefly, carefully remove and discard <u>all</u> the supernatant.

3) Resuspend the cells in 50 µl 1 X PBS and 5 mM EDTA

4) Lyse the cells by freezing and thawing 3 times.
Freeze the cells at -80 °C for 5 min. / Thaw at RT 5 min., then vortex / Repeat 3x

5) Pellet the debris in the microfuge — 3-4 min at full speed.
Use the supernatant for CAT assays.
Store tube (super + pellet) @ -20° C. Pellet debris before using.

<u>CAT ASSAY</u>

Always include controls of
i) Negative control (uninfected cells)
ii) Positive control (0.05-0.1 unit Sigma CAT enz. added)
iii) No-cell extract control

6) Add 25-30 µl of the cell extract (80 ug of protein as determined by Bradford Protein Assay to a glass mini-scintillation vial.

7) Add 230 µl of the MASTER MIX. Mix by swirling gently

MASTER MIX

1 rxn	10 rxns	
194.5 µl	1.945 ml	H_2O
23 µl	230 µl	1 M Tris (pH 7.8)
2.5 µl	25 µl	Chloramphenicol (100 mM in EtOH)
10 µl	100 µl	^{14}C-Acetyl Co-A (NEN,0.01 µCi/µl
230 µl	2.3 ml	or 0.1 µCi/rxn)

8) Gently overlay with 5 ml of Econofluor (NEN).

9) Incubate at 37 ^{o}C for 1 hour before counting.

10) Count each sample for 1 minute at 1-2 hour intervals.

Reagents:
1 M Tris (7.8) — sterile
^{14}C-Acetyl Coenzyme A (NEN Catalog #NEC-313L)
Chloramphenicol (Sigma): Make a 100 mM solution in EtOH as per Maniatis. Store at -20^{o} C
Econofluor NEN Cat. No. NEF-941 or formula 989 = Econofluor NEF-989
Glass Mini-scintillation vials (VWR) — 7 ml capacity
mini-vial holders — plastic
Rubber policeman (clean and sterile) — 1 per well

References:

J. R. Neumann, C. A. Morency, and K. O. Russian (1987). A novel rapid assay for chloramphenicol acetyltransferase gene expression. Biotechniques (1987) Vol. 5, No. 5, p 444-447.

NEN pamphlet supplied with ^{14}C-Acetyl Co-A contains a more detailed account of the procedure with tips and recommendations.

Appendix 10: Frozen Competent Cells $CaCl_2$ and DMSO Procedures

Culturing of Bacteria:

1) Inoculate 200 ml of Luria broth with 2 ml of a fresh overnight culture (6-14 hour culture in log phase growth).

2) Incubate at 37 °C for approximately 1.5 — 2 hours until the OD_{600} reaches 0.3-0.4.

3) Pellet cells in 250 ml bottle in Superspeed (Sorvall) centrifuge(4000 rpm for 5 min). Discard supernatant. Complete either the FCC $CaCl_2$ or DMSO procedure.

Frozen Competent Cells $CaCl_2$ Procedure:

1) Resuspend the cells **gently** in 100 ml of sterile ice-cold 50 mM $CaCl_2$, 10 mM Tris (pH 8.0) (1/2 original volume).

Place the resuspended cells on ice for 15 mins.

2) Pellet cells in Servall centrifuge (4000xg for 5 mins).

Discard supernatant.

3) **Gently**, resuspend the cells in 13 ml of Sterile ice cold 50 mM $CaCl_2$, 10 mM Tris (pH 8.0)(1/15 the original volume). Place the resuspended cells on ice for 10 mins. before using. (The cells may be stored on ice at 4 °C for 48 hours without ill effect. Transformation effeciency increases at 24 hrs. and decreases to the original efficiency at 48 hrs.)

4) To freeze the cells after 24 hrs. on ice, add cold Sterile Glycerol to a final concentration of 15% (2.8 ml 80% glycerol + 13 ml cells). Mix by gently pipeting with a 5 or 10 ml sterile pipet.

Place on ice for 10-15 mins.

5) Aliquot 300-700 µl per 1.5 ml epp tube and immediately place tubes on ice. Precool pipet tips at 4 °C prior to pipeting. Tubes should be pre-chilled by placing at -80 °C for approximately 30 mins.

6) Store tubes at -80 °C.

Transformation Using Frozen Competent $CaCl_2$ Cells:

1. Thaw a tube of FCC in ice water bath (app. 2-4 mins). Place on ice.

2. Add DNA (ligation or other transforming DNA) to a sterile 17 x 100 mm culture tube. Add 300 µl of FCC to each tube and mix. If you are transforming a low melt agarose ligation do not place on ice until the DNA and FCC are mixed.

When testing the effiency of frozen competent cells add 10 µl of a 10 ng/ml stock of pBS DNA.

3. Incubate on ice for 45 mins.

4. Heat shock cells in a 42 °C waterbath for 90 sec.
   Place the cells on ice for 2-3 mins.

5. For plasmid cloning, add 4 volumes SOC to the FCC (i.e. 1200 µl of SOC to 300 µl of FCC)
   Incubate in a 37 °C shaker for 1 hour.

Plate varying quantities of the culture on plates containing the appropriate antibiotic and perhaps IPTG and Xgal.
IPTG - 50 µl of 20 mM IPTG spread on the surface of the plate
XGal - 50 µl of 2% XGal (in N-N-Dimethyl formamide) spread on the surface of the plate.

Allow 14-16 hours for blue color to develop.
Note: JM83 and DH5α cells do not require that IPTG be added.

Frozen Competent Cells DMSO Procedure:

1. Gently resuspend pelleted bacteria in 1/10th volume of 1 X TSS on ice.

2. Aliquot resuspension into prechilled 1.5 ml eppendorfs and freeze at -80 °C.

Transformation and Storage Solution (TSS)
1 X TSS is LB media containing 10% (wt/vol) polyethylene glycol, 5% (vol/vol) dimethyl sulfoxide (DMSO), and 50 mM $MgSO_4$ at pH 6.5.

Transformation DMSO Frozen Competent Cells (FCC):
1. Thaw a tube of FCC in ice water bath (app. 2-4 min), then place on ice.

2. Add DNA (ligation or other transforming DNA) to a sterile 17 x 100 mm culture tube. Add 300 µl of FCC to each tube and mix. If you are transforming a low melt agarose ligation, do not place on ice until the DNA and FCC are mixed.

3. Incubate on ice for 45 mins. If you are simply retransforming circular DNA, 5 min on ice is sufficient.

4. For plasmid cloning, add 4 volumes SOC to the FCC (i.e. 1200 µl of SOC to 300 µl of FCC). Incubate at 37 °C in a shaker for 1 hour.

5. Plate varying quantities of the culture on plates containing the appropriate antibiotic and perhaps IPTG and Xgal. If desired, cells may be tranferred to a 1.5 ml eppendorf and centrifuged to concentrate cells before plating. I usually plate a ligation on three plates at the following percentages 5%, 25%,and 70%.

SOB Medium:

2% Bacto Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$.

SOB medium is prepared without Mg and autoclaved. After cooling, add 1/100 vol. of sterile 1 M $MgCl_2$ and 1/100 vol. of sterile1 M $MgSO_4$.

SOC Medium:

SOC is SOB + 20 mM glucose. Make a 2 M glucose solution in H_2O and sterile filter it. Use good sterile technique as the glucose and SOC solutions are easy to contaminate. Add 1/100 vol of the 2M glucose to SOB.

Calculations for Transformation Efficiency:

If 300 µl of cells are transformed with 100 ng of pBS DNA, the following calculations apply: Final concentration of DNA = 100 ng in 1.5 ml Therefore, DNA = 0.066 ng/µl

If 300 µl of cells are transformed with 100 ng of plasmid DNA, plate out the following amounts: 1.5 µl, 15 µl, 150 µl and use the table below to determine efficiencies.

Amount plated	# colonies expected at this efficiency			
	$1x10^5$	$1x10^6$	$1x10^7$	$1x10^8$
1.5 µl = 0.1 ng	10	100	1,000	10,000
15 µl = 1 ng	100	1,000	10,000	100,000
150 µl = 10 ng	1,000	10,000	100,000	1,000,000

$> 1 \times 10^7$ = good to excellent

1×10^6 to 1×10^7 = acceptable

1×10^5 to 1×10^6 = usable in a bind but not good

$< 1 \times 10^5$ = Discard and try again

Reference

DMSO procedure: Chung, C.T., Niemela, S.L., and Miller, R.H. (1989) One-step preparation of competent Escherichia coli: Transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. 86; 2172-2175.

Appendix 11: Site-Directed Mutagenesis Procedure

TITER HELPER PHAGE M13KO7

1) Prepare a 25 ml culture of MV 1190 (or any other strain that contains an f-plasmid) in LB and incubate at 37 ^{o}C with shaking overnight.

2) Prepare 5 tubes of top agar by melting it in the microwave oven and pipet 3 ml of agar into sterile tubes. Keep at 50-55 ^{o}C until use.

3) Prepare 4 serial dilutons of the M13KO7 stock (10^{2}-fold, 10^{4}-fold, 10^{6}-fold, 10^{8}-fold) in sterile tubes in LB. For example: 5 µl of M13kO7 stock in 495 µl of LB (10^{2}-fold) and so on.

4) Place 0.2 ml of the overnight culture of MV 1190 into another 4 sterile tubes. Add 100 µl of the last three dilutions into one of these tubes (the fourth will serve as a control).

5) After 5 minutes at room temperature, pipet the contents of each tube containing the cells and phage into a tube of top agar. Pipet up and down (Do not introduce any bubbles). Plate IMMEDIATELY ONTO AN LB PLATE.

6) Allow the top agar to harden for 15 mins, then invert the plates and incubate at 37 ^{o}C overnight.

7) The following morning, count the plaques on the plate that has a countable number on it. Use the following formula to get the titer:

The titer =(# of plaques x 10 x dilution factor)pfu/ml

TEMPLATE PREPARATION

1) An F1 origin containing plasmid that contains the sequence to be mutagenized should be transformed in either CJ 236 (ung^-, dut^-) or RZ1032 (ung^-, dut^-).

2) Prepare a 10 ml overnight culture from one colony in 2XYT. When CJ 236 cells are used, grow in 50 μg/ml of AMP and 30 μg/ml of Chloramphenicol. When RZ 1032 cells are used, grow in 50 μg/ml of AMP and 12.5 μg/ml of Tetracycline.

3) In a 250 ml flask, inoculate 100 mls of 2XYT and 50 μg/ml of AMP only with 2 ml of overnight.

4) Grow to OD_{600} of 0.3 ABS (1 to 4 hours)

5) This represents 1×10^7 cell/ml. Add enough M13kO7 helper phage to obtain 20 phage/cell.

6) Incubate in shaker at 37 °C for 1 hour. Add 233 μl of a 50 mg/ml Kanamycin stock per 100 ml of media.

7) Continue to incubate 18 to 20 hours.

8) Transfer culture to 4 corex tubes (25 mls/tube) and spin at 10 K for 15 mins

9) Repeat step 8, transfer Supernatant to a new sterilized corex tube and spin at 10 K for 15 mins

10) Transfer 20 mls of the Supernatant to a fresh corex tube and add 100 μg of RNase A or 10 μl of 10 mg/ml stock. Incubate this mixture at room temperature for 30 mins

11) Add 2.5 mls of 7.5 M NH_4OAc and 2.5 mls of 40% PEG 8000 to each 20 ml of supernatant. Incubate on ice 30-45 mins.

12) Centrifuge 10 K for 30-60 mins. Decant off supernatant, resuspend each 25 ml tube pellet in 150 µl of High salt buffer.

High salt buffer
300 mM NaCl
100 mM Tris pH 8.0
1 mM EDTA

Transfer to a 1.5 ml Eppendorf tube
Chill on ice for 30 mins

Centrifuge for 2 min and transfer to a fresh tube

13) May be stored overnight at 4 ^{o}C (should not store longer than a couple of days) or extracted immediately

14) The 600 µl of high salt buffer should be extracted:

1 X phenol (add half volume)
1 X phenol / chloroform (add 1/4 volume of each)
1 X chloroform (add 1/2 volume)

15) Add 1/ 10 volume of 7.5 M NH_4OAc and 2.5 volume of 95 % ethanol (60 µl and 1320 µl respectively). Need to split into two 1.5 ml eppendorf tubes.

16) Keep at - 80 ^{o}C at least 30 mins

17) Centrifuge in the cold for 15 - 30 mins. Remove supernatant carefully and resuspend the pellet(s) in a total of 50-100 µl of TE pH 8.0. Avoid dissolving any residue that may cling to the side of the tube.

18) Transfer the DNA to a new tube. Run a small aliquot on an agarose gel with a known amount of single-stranded DNA to determine the concentration.

PHOSPORYLATION OF THE MUTAGENIC OLIGONUCLEOTIDE

1) In a sterile 500 µl tube prepare the following reaction:

Component:	**Volume added**	**Final concentration**
Oligonucleotide 200 pmol/µl	10 µl	66.6 pmol/µl
1 M Tris pH 8.0	3 µl	100 mM
0.2 M $MgCl_2$	1.5 µl	10 mM
0.1 M DTT	1.5 µl	5 mM
10 mM ATP	1.3 µl	0.4 mM
DDH_2O	12.7 µl	
Total	30 µl	

2) Add 4.5 units of T4 Polynucleotide Kinase

3) Mix and incubate at 37 °C for 45 mins

4) Stop reaction by heating at 65 °C for 10 mins

ANNEALING OF THE MUTAGENIC OLIGONUCLEOTIDE TO THE TEMPLATE

1) In 500 µl tube prepare the following reaction mix:

Component	Volume added	Final Concentration or Mass
Uracil containing DNA		0.3 pmoles
Kinased mutagenic oligo		9.0 pmoles
10X Annealing Buffer	3 µl	20 mM Tris pH 7.4 2 mM $MgCl_2$ 50 mM NaCl
DDH_2O	----------	
TOTAL VOLUME	30 µl	

The optimum template to mutagenic oligo ratio is between 1:20 or 1:30

Useful formulas for calculating ratios

$$\text{template } (\mu g \text{ or } ng) \times \frac{(\#\text{ of bases in oligo})}{(\#\text{ of bases in template})} = \text{amount } (\mu g \text{ or } ng) \text{ of oligo needed for 1:1 ratio.}$$

Molecular weight of base = 330 g/mole or 3.30×10^{-4} μg/pmole
To determine the MW of the oligo or the template:

# of bases x 3.3 x 10-4 μg/pmole = Molecular Weight (μg/pmole)

2) Prepare an identical tube containing No primer. Controls for nonspecific endogenous priming.

3) Place reaction mixtures in a 70 °C water bath for 3 mins. Allow the reaction mixture to cool in the water bath at a rate of 1 °C/min to 30 °C

4) Place reaction on ice for 2-3 mins.

SYNTHESIS OF COMPLIMENTARY DNA STRAND

1) Add the following to each annealing reaction (Keep tubes on ice as much as possible throughout this step)

10 X Synthesis buffer	3 μl	0.4 mM each dNTP 0.7 mM ATP 17.5 mM Tris HCl pH 7.4 3.75 mM $MgCl_2$ 1.5 mM DTT
T4 DNA Ligase (1 unit/μl)	2 μl	
Sequenase diluted 1:4 (3.25units/μl)	1 μl	
OR USE		
Klenow diluted to 5 units/μl	1 μl	

2) Allow reactions to sit on ice for 5 mins after adding components.

If Sequenase is used:

1) Transfer the reactions to 25 °C for 5 mins; then to 37 °C for 90 mins.

OR

2) Transfer reactions to 15 °C water bath for 8 hours to overnight.

If Klenow is used:

Transfer reactions to 15 °C water bath for 8 hours to overnight.

Transform about 10 µl of each of the *in vitro* reactions with competent cells and plate.

Appendix 12: Total Cell RNA Isolation using CsCl Cushion

Harvesting RNA Samples

Do all samples individually through step 3

1) Aspirate off cell culture media

2) Rinse culture dish with 1 x PBS and aspirate off PBS

3) **Immediately** place dish at -80 °C on cold melt surface. Keep dishes stored at -80 °C until needed.

Isolating RNA Samples

Work in the hood

Do all samples individually through step 6

4) Remove one sample at a time from the -80 °C freezer and immediately place 250 µl of Guan-Thio solution in each of the 6 well plate. Scrape up all cells into this solution. Do not thaw out the plate.

5) Place the sample in a 15 ml disposable centrifuge tube that contains 0.6 g of solid CsCl. Invert to dissolve and adjust volume to 1.5 ml with Guan-Thio (if needed).

6) Sonicate homogenate for 30 sec at maximum with small sonicator probe.

7) Layer homogenate on a 1.4 ml CsCl pad (5.7 M CsCl, 0.1 M EDTA) in a 3 ml heat seal tube (use a 3cc syringe 18.5 gauge needle to load on the CsCl pad and a P-200 pipetman tipped with a sequencing tip to load on the homogenate).

8) Centrifuge at: 49k rpm in Beckman 100.3 rotor (110,000g) using a Beckman Tabletop Ultracentrifuge (TL100) for 16-20 hours at 25 °C.

Work quickly and process samples individually through step 12

Work in the hood

9) Cut off the top of the tube and remove the supernatant above the pad with a sterile pasteur pipet.

10) Cut off the top portion of the tube, with a pastuer pipet remove the floating cell pellet and the Guan-Thio layer. Pour off the CsCl pad and allow the tube to drain. RNA is at the bottom and looks like a contact lens.

11) Take up the clear, contact lens-like pellet in 200 µl of 1X TES. Rinse with a another 200 µl of 1X TES. (If pellet does not dissolve completely add another 400 µl of 1X TES and split the sample into two 1.5 ml eppendorf tubes.) Transfer all 400 µl to a tube containing 100 µl of phenol (water saturated) and 100 µl of Chloroform (24:1 $CHCl_3$: Isoamyl alcohol). Vortex vigorously for 1 min.

12) Microfuge and remove aqueous phase to a fresh tube containing 200 µl of Chloroform. Vortex and microfuge.

13) Remove aqueous to a fresh tube and add 50 µl of 3M NaAc pH5.2 and 1000 µl of 100% Ethanol (RNA Grade, stored in -20 ^{o}C).

Gaun-Thio

Component	Stock	Recipe
4 M guanidinium isothiocyanate		25.0 g
0.1 M 2- mercaptoethanol	12.8 M	414 µl
5 mM sodium citrate pH 7	1 M	265 µl
0.5% sarcosyl	10%	2.65 ml
Milli-Q water		27 ml
Total		53 ml

10 X TES

Component	Stock	Recipe
10% SDS	20%	25 ml
Milli-Q water	15 ml	
Boil to sterilze, then add sterile ingredients below		
0.1 M Tris pH 7.5	1 M	5 ml
50 mM EDTA	0.5 M	5 ml

Appendix 13: Primer Extension Procedure

5' End Labeling the Oligonucleotide
(T4 Polynucleotide Kinase)

X µl of H_2O
2.0 µl of 10X Kinase Buffer
X µl of Oligonucleotide (300 ng, ≥ 100 ng)
X µl of ^{32}P-gamma-ATP (50-100 µCi @ 6000 Ci/mmol)
X µl of Polynucleotide Kinase (≥ 7 units)
20 µl

Incubate @ 37 °C for 30 min.
Incubate @ 65 °C for 5 min.
Store at -20 °C
Final Concentration = 5 ng/µl

Use 1 µl (5 ng) for annealing to RNA

Annealing Oligo to RNA

1. Dry down RNA samples (if needed) using a Speed Vac. Use 1.5 ml eppendorf tubes

2. Mix 1 µl (5 ng) of ^{32}P-labeled
X µl of (20-50 µg) total or cytoplasmic RNA
X µl of H_2O
1.5 µl of 10X Annealing Buffer
15.0 µl

3. Heat @ 70 °C for 3 min.
4. Allow primer to anneal 45 min @ X °C.

X = ≈ 5 °C below the denaturation temp (T_d) [can be 2-3 °C below)
$T_d = 4(G+C) + 2(A+T)$
T_d is the temperature at which 1/2 of the primer:template complexes are disassociated and is in °C

Primer Extension Reactions:

Using MMLV Reverse Transcriptase
To each 15 µl of Annealing Rxn add 30 µl of PE MIX

PE Mix:	4.5 µl	10x MMLV RT buffer (GWB)
	1.5 µl	MMLV RT (200 units/µl)
	24.0 µl	DDH_2O
	45.0 µl	total

Incubate @ X °C or 50 °C for 30-45 mins

Using AMV Reverse Transcriptase
To each 15 µl of Annealing Rxn add 30 µl of PE MIX (keep samples at annealing temp)

PE Mix:	4.5 µl	10X RT Mix (see below)
	X µl	H_2O
	X µl	AMV Rev.Transcriptase (Promega; app 5 units/rxn)
	45 µl	Total Volume

Incubate @ Annealing Temp "X" for 45 min
Note: If "X" is > 50° C , use 50 ° C.

AFTER EXTENSION REACTION DO A or B:

A. If desired RNAse treat as folows:
To each tube add:
1 µl of 0.5 M EDTA, Mix
1 µl of RNAse (1 mg/ml; DNAse free)
Incubate at 37 °C, 20 min. Stop the P.E. rxn;

Stop the P.E. rxn ; EtOH ppt.
5 µl of 3 M NaAc pH 5.2, **mix**
100 µl of EtOH

Incubate at -80 °C for 20 min or @-20 °C for 1hr - overnight
Pellet PE rxns 30 min in a microfuge. Carefully aspirate supernatant.
Remove **all** EtOH.
Resuspend each pellet in 5 µl of Formamide Sample buffer or Sequenase STOP Buffer. Heat to 80 °C and load on a gel

B. Alkaline treat as follows (recommended)
To each tube add:

5 µl	of 3M NaAc pH 5.2,	mix
100 µl	of EtOH	

Incubate at -80 °C for 20 min or @ -20 °C for 1hr - overnight

Spin, resuspend in 6 µl of 0.1M NaOH and 1 mM of EDTA ; Make sure the RNA is properly dissolved.
Incubate at 30 °C for 30 min
Add to each tube, 6 µl of Formamide Sample buffer or Sequenase STOP Buffer
Heat to 80 °C and load on gel

10X Kinase Buffer:
500 mM Tris (7.4), 100 mM $MgCl_2$, 50 mM DTT, 1 mM Spermidine

6.05 g	Tris
2.03 g	$MgCl_2$
0.771 g	DTT
0.025 g	Spermidine
To 100.0 ml	w/ H_20; pH to 7.4; Filter Sterilize

10X Annealing Buffer:
2.5 M KCl, 100 mM Tris (8.3) (pH 9.0 @ 25 °C)

Concentration	Component	Stock	Volume	Final
2.5 M	KCl	4 M	625 µl	250 mM
100 mM	Tris	1 M	100 µl	10 mM
MQ Water			275 µl	
Filter Sterilize				

10X Extension Buffer (GWB)

225 mM Tris (pH 9.0 @ 25 °C), 400 mM KCl, 50 mM MgCl, 50 mM DTT, 2.5 mM dNTP's

Concentration	Component	Stock	Volume	Final
225 mM	Tris	1 M	50 µl	25 mM
400 mM	KCl	4 M	20 µl	40 mM
50 mM	MgCl	1 M	10 µl	5 mM
50 mM	DTT	100 mM	100 µl	5 mM
2.5 mM	dNTP's	100 mM	5 µl	0.25 mM
	(5 µl of each dNTP for a total of 20 µl)			
Total volume			200 µl	

5X MMLV RT BRL BUFFER (supplied)

250 mM Tris-HCl pH 8.3
375 mM KCl
50 mM DTT
15 mM $MgCl_2$

10X AMV RT Mix

0.5 M Tris (8.0), 0.5 M KCl, 50 mM $MgCl_2$, 50 mM DTT,
2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dTTP, 2.5 mM dGTP,

1. Prepare a large stock of 10 RT Mix without dNTPs (app. 30-50 ml)
0.556 M Tris (8.0), 0.556 M KCl, 55.55 mM $MgCl_2$, 55.55 mM DTT,

2. Prepare smaller aliquots of the complete 10X RT Mix by adding a dNTP mix.

1.35 ml of 10X RT Mix without dNTPs
0.15 ml of dNTP Mix (25 mM each dNTP)
1.50 ml 10 RT Mix
(aliquot to 5 tubes @ 300 µl/tube)
Store at -20 °C

Formamide Sample Buffer

100% Formamide w/ 0.3% Bromophenol Blue and 0.3% Xylene Cyanol

Appendix 14: β-Galactosidase Assay

24 well plate assay

For recombinant virus assay

1) Plate 1.5 x 10^6 cells/well and allow cells to attach for at least one hour.
2) Dilute the amount of virus used (MOI) for each well in 800 µl of media. Always calculate (virus and media) for a few extra wells.
3) Remove media and place the 800 µl of media containing virus in each well. Incubate for one hour.
4) After one hour (called time zero), remove the 800 µl and replace with 1 ml of fresh media.

For transient expression assay (start here)

5) After the desired incubation time, scrape cells loose of 6 well plate with policeman.
6) Transfer to 1.5 ml eppendorf tubes.
7) Spin cells for 3 mins at 60% in microfuge.
8) Take off supernatant and respin briefly to remove any remaining supernatant. Add 500 µl of 1 x PBS, vortex pellet in solution and freeze at -80 °C.
9) Thaw cell extracts.
10) Centrifuge to pellet cell debris. If desired, transfer supernatant to a fresh tube and store at -80 °C.
11) Make at least two identical controls wells, they will be used to zero the spectrophotometer. Choose from one of two methods: a) replace the 5 µl of cell extract with 5 µl of 1 x PBS. b) Use a 5 µl of a cell extract that has not seen a *lac* Z construct.
12) Place 5 µl of cell extract in each well and incubate at 37 °C for 5 mins.
13) Add quickly 995 µl of Buffer Z in 24 well plate mix, and place at 37 °C for 5 mins.
14) Then add 200 µl of ONPG, mix and incubate at 37 °C.

To quantitate:
For recombinant virus: incubate for 5 to 10 mins and begin taking timepoints every 5 to 10 mins. Take at least 4 timepoints.
For transient expression assay: incubate for 30-60 mins and begin taking timepoints every 30-60 mins. Take at least 4 timepoints.
15) At each timepoint remove 200 µl of the assay and transfer to a 1.5 ml eppendorf containing 800 µl of 0.5 M Na_2CO_3.

96 well plate assay

1) Pipet up and down several times and then remove 80 µl of sample to a new 96 well plate. Store the remainder at 4 °C.
2) Freeze and thaw at -80 °C one time.
3) Add 120 µl of Buffer Z (pipet up and down to mix)
4) Incubate 5 mins at 37 °C
5) Add 40 µl of ONPG (pipet up and down to mix)
6) Wrap plates in foil and incubate at 37 °C for 2 hours to overnight.
7) Stop reaction by addition of 50 µl of 1 m Na_2CO_3

Solutions

Buffer Z 100 mls:

1.61 g Na_2HPO_4 $7H_2O$ (0.06M)
0.55 g NaH_2PO_4 H_2O (0.04M)
0.075 g KCl (0.01M)
0.024 g $MgSO_4$ $7H_2O$ (0.001M)
0.27 ml 2-Mercaptoethanol
Stored at room temperature in a tightly sealed bottle good for 1 to 2 months

1 M Na_2CO_3: 4.2 g/40 ml
0.5 M Na_2CO_3: 2.1 g/40 ml
Stored at room temperature good for 1 week

ONPG:

ONPG 4 mg/ml in 0.1 M $NaPO_4$ (pH 7.0)
Store at -20 °C and wrap in foil.

Appendix 15: Non-CsCl Method To Isolate Total Cell RNA

1) Plan to work in the Hood! To harvest RNA remove media from one well at a time and add 400 µl of 1 x PBS to rinse (gently rock plate back and forth). This procedure is scaled for 1 to 1.5 X 10^6 cells/well
2) Remove all liquid from cells
3) Add 600 µl of solution D to the well and use a rubber policeman to scrape loose the gelatinous cell debris. Transfer to an eppendorf.
4) Return to step 1 and harvest the next well; after harvesting all wells, add 60 µl of 2 M Sodium Acetate pH 4.0. Mix by vortexing.
5) Add 600 µl of water-saturated phenol and mix by vortexing.

(Note Phenol should be nucleic acid graded and equilibrated with only RNA Grade water. Do not use Tris equilibrated phenol)

6) Add 120 µl of Chloroform mix by vortexing for 10 sec. Cool on ice for 15 mins.
7) Centrifuge samples at 10,000g for 30 mins at 4 °C
8) Transfer aqueous phase to fresh eppendorf and add 600 µl of isopropanol. Place at -20 °C for at least an hour to precipitate the RNA.
9) Centrifuge at 10,000g for 20 mins at 4 °C
10) Resuspend RNA pellet in 100 µl to 300 µl of solution D and mix.
11) Reprecipitate with 1 volume of isopropanol. Leave at -20 °C 1 hour.
12) Centrifuge for 20 mins at 4 °C
13) Wash RNA pellet with 75% ETOH and pellet
14) Vacuum dry (15 mins) and dissolve in 50 µl of RNA GRADE WATER.
15) Read the OD_{260} to determine the concentration

$$\text{Concentration} = \frac{(OD260)\,(\text{dilution factor})(50\ \mu g/\mu l)}{1000}$$

REAGENTS

Solution D : 4 M guanidium thiocyanate, 25 mM Sodium Citrate pH 7.0, 0.5% Sarcosyl, 0.1 M b-Mercaptoethanol.
2 M Sodium Acetate pH 4.0
Phenol (Water-Saturated only)

Appendix 16: Taq Sequencing Procedure

1) DNA to be sequenced should be prepared as described in the DNA Mini-prep protocol. For best results the DNA should be used within one week. Prepare four 0.5 ml termination tubes labeled A,C,G,T for each sample and add 1 µl of the proper deazo dNTP to each tube. Store on ice.

2) Use 0.5 ml eppendorf tube and add the following components:

44 µl of a Mini-prep DNA resuspended in 100 µl of water or TE
5 µl of 2mM EDTA 2 M NaOH
49 µl

3) Heat 5 mins at 85 °C

4) Place on ice 3 mins

5) Add:

6 µl of 3 M NaAc pH 5.2 mix
150 µl of Ethanol

6) Place at -80 °C for 15 mins

7) Spin for 20 mins in the microfuge at 4 °C.

Remove supernatant, respin and carefully remove remaining supernatant with a sequencing tip.

8) Add to each sample:

15.5 µl of MQwater
2.5 µl of primer 10 ng/µl
5.0 µl of 5X Taq polymerase buffer
2.0 µl of extension/labeling mix
25.0 µl

9) Anneal at 37 °C 25 mins. Remove and cool to room temperature slowly.

10) Add to each sample:

2.0 µl of ^{35}S-dATP
1.6 µl of Taq polymerase

11) Incubate at 37 °C for 5 mins

12) Add 6 µl of the reaction to each of the four terminantion tubes.

13) Incubate at 70 °C for 17 mins

14) Add 4 µl of stop solution to each termination tube. Store at -20 °C

Appendix 17: RNase Protection Procedure

In vitro RNA Synthesis

1) Linearize 20 µg of CsCl purified plasmid (digest at 0.1 µg/ul of plasmid DNA) overnight with the appropriate restriction enzyme. Verify that the digestion is complete by loading on a gel. 5' overhangs are best, blunts are marginal and 3' overhangs are poor linearization sites. 3' overhangs allow polymerase to turn around and synthesize back on the non-coding strand.

2) Phenol/Chloroform extract, then Chloroform extract. Ethanol precipitate using 1/10 volume of 3 M NaAc and 2 volumes of ethanol. Precipitate at -80 °C for 20 mins.

3) Microfuge 30 mins at 4 °C and remove supernatant. Respin and remove remaining supernatant. Resuspend linearized plasmid DNA in water at 0.5 µg/ul. Store at -20 °C until needed.

4) Assemble the transcription reaction in a 1.5 ml eppendorf tube.

Component	Stock	Volume
2 µg of linearized plasmid	0.5 µg/µl	4 µl
1x Transcription buffer	5x	4 µl
10 units of RNasin	40 units	0.4 µl
10 mM of DTT	100 mM	2 µl
50 µM of Cold UTP	1 mM	2 µl
50-60 µCi of ^{32}P UTP	10 µCi/µl	5 µl
0.5 mM of rGTP rCTP rATP	5 mM each	2 µl
Polymerase (T7,T3 or Sp6)	20 units/µl	1 µl
MQ Water		1 µl
		20 µl

5) Incubate at 37 °C for 1 hour.

6) Add 1.5 µl of RNasin 40 units/µl
3.0 µl of Yeast RNA 10 ug/µl
0.6 µl of DNase (free of RNase) 5 units/µl

7) Incubate 37 °C for 30 mins, then add 175.5 µl of 1x TES. Remove 1 µl and dilute in 99 µl of MQ water. Add 5 µl to a scintillation vial and save (Crude input counts Diluted 20X). Store sample at -20 °C.

8) Prepare column using a 5 ml disposable pipette cut off at -2 ml mark and plug with baked glass wool. Fill column with G-50/1x TES.

9) Load the remaining 199 ul of the reaction onto the column that has no buffer over the gel bed. After the sample has been absorbed onto the column, add more buffer. Do not let the column dry out. Monitor the separation of the unincorporated vs the incorporated counts with the gieger counter.

10) Collect the first peak (incorporated counts usually come off in a 1 ml volume) in a 1.5 ml eppendorf (split into four 1.5 ml eppendorfs). Precipitate by adding 1/20 volume of 5 M NaCl and 3 volumes of 95% ethanol. Store at -80°C for 2 hours. Microfuge for 30 mins, remove supernatant, respin, remove remaining supernatant. Resuspend in 100 to 150 µl of water. Remove 1 µl and dilute in 99 µl of water. Then add 5 µl to a scintillation vial and save (diluted 20X). Store at -20 °C.

11) Calculate the mole of RNA synthesized:

$$\text{Compute \% incorporation} = \frac{\text{pure counts} \times 20}{\text{crude counts} \times 20}$$

12) Compute pmoles of UTP (hot plus cold) and add to the transcription reaction.

$1/(800\ \text{Ci/mmole} \times 10^3\ \text{mmole/mole})\ 50\times10^{-6}\ \text{Ci}$
$= 6.25\times10^{-11}$ mole

$6.25\times10^{-11} + 1.0\times10^{-10} = 1.625\times10^{-10}$ moles = 160 pmole

13) $$\text{pmol probe} = \frac{(\text{pmol input UTP})(\%\ \text{inc.}/100)(4\ \text{NTP/UTP})}{\text{probe length}}$$

$\text{pmol} = \text{ug}/(0.66)(\text{kb})$

$\text{mole} = \text{pmol}\ 10^{-12} = \text{fmol}\ 10^{-15} = \text{attomol}\ 10^{-18}$

Solutions

5X Transcription Buffer
0.2 M Tris pH 7.5
30 mM $MgCl_2$
10 mM spermidine

1x TES

10 mM Tris pH 7.5	Tris 0.5 ml of 1 M
5 mM EDTA	EDTA 0.5 ml of 0.5 M
1% SDS	

RNase protection assay

Make up standards and controls
Tubes 1-5 are standards
1) 6000 attomoles ψ mRNA 5 µl + 5 µl 10 µg/µl yeast RNA
2) 1600 attomoles ψ mRNA 5 µl + 5 µl 10 µg/µl yeast RNA
3) 400 attomoles ψ mRNA 5 µl + 5 µl 10 µg/µl yeast RNA
4) 100 attomoles ψ mRNA 5 µl + 5 µl 10 µg/µl yeast RNA
5) 20 attomoles ψ mRNA 5 µl + 5 µl 10 µg/µl yeast RNA

No protection control
6) Just probe and 10 µl of 5 µg/µl yeast RNA

No digestion control
7) Just probe and 10 µl of 5 µg/µl yeast RNA. Leave out of RNase digestion

A) Prepare samples in 1.5 ml eppendorfs using 30 µg/µl of total RNA and 20 µg/µl of yeast RNA. Dry down samples.

B) Add Hybridization mix (volume 30 µl) to each sample.

Hybridize at 55 °C for 12-15 hours.

Hybridization mix
24 µl of 100% formamide
3.7 µl of Hybridization buffer
x µl of probe 5 fmoles (max volume 2.3 µl)
x µl of water
30 µl Total

Hybridization buffer (25 samples)

Component	Stock	Volume	Final Concentration
NaCl	5 M	80 µl	0.4 M
EDTA	0.5 M	2 µl	1 mM
Pipes pH 7.0 @ 25 °C Sigma P-6757	1 M	40 µl	40 mM

C) Centrifuge briefly
Add 300 µl of RNase mix to each sample.
samples/3 = # mls of RNase mix

0.3x RNase Mix ** (make fresh)

Volume	Component
1.0 ml	1x RNase Digestion buffer
1.0 µl	10 mg/ml RNase A
1.0 µl	350 units/µl RNase T1

** Just add RNase Digestion buffer to standard tube # 7

Cap tubes, Flick several times to spread RNase to all surfaces of the tube. Microfuge briefly

Place at 37 °C for 1 hour

1x RNase Digestion Buffer

Concentration	Component	Stock	Volume
10 mM	Tris pH 7.5	1 M	100 µl
5 mM	EDTA	0.5 M	100 µl
300 mM	NaCl	5 M	600 µl
	Water		9.2 ml
			10.0 ml

D) Add 20 µl of Proteinase K Digestion mix
Proteinase K mix
50 µl of 20 mg/ml proteinase K
50 µl of 10 mg/ml Yeast RNA
900 µl 10X TES
1000 µl

Incubate 15 mins at 37 °C

E) Extract one time with phenol/chloroform, then Ethanol precipitate
Add:
40 µl of 3 M NaAc pH 5.2
880 µl of 100% Ethanol -80 °C 1 hour or -20 °C overnight

F) Centrifuge 30 mins and resuspend in 10 µl of a mixture (2 µl of water and 8 µl of loading buffer). Be sure samples are resuspended.
Store at -20 °C.

Appendix 18: GUS Assay

24 well plate assay

For recombinant virus assay

1) Plate 1.5 x 10^6 cells/well and allow cells to attach for at least one hour.
2) Dilute the amount of virus used (MOI) for each well in 800 µl of media. Always calculate (virus and media) for a few extra wells.
3) Remove media and place the 800 µl of media containing virus in each well and incubate for one hour.
4) After one hour (called time zero) remove the 800 µl and replace with 1 ml of fresh media.

For transient expression assay (start here)

5) After the desired incubation time, scrape the cells loose of 6 well plate with policeman.
6) Transfer to 1.5 ml eppendorf tubes.
7) Spin cells for 3 mins at 60 % in microfuge.
8) Take off supernatant and respin briefly to remove any remaining supernatant. Add 500 µl of 1 x PBS, vortex pellet in solution and freeze at -80 °C
9) Thaw cell extracts.
10) Centrifuge to pellet cell debris. If desired transfer supernatant to a fresh tube and store at -80 °C.
11) Place 5 µl of cell extract (for recombinant virus) or 50 µl (for transient assay) to each well and incubate at 37 °C for 5 mins.
12) Add quickly 595 µl(for recombinant virus) or 600 µl (for transient assay) of GUS assay buffer in 24 well plate and place at 37 °C.

To quantitate:

For recombinant virus: incubate for 5 to 10 mins and begin taking timepoints every 5 to 10 mins. Take at least 4 timepoints.

For transient expression assay: incubate for 30-60 mins and begin taking timepoints every 30-60 mins. Take at least 4 timepoints.

13) At each time point, remove 100 µl of the assay and transfer to a 1.5 ml eppendorf containing 900 µl of 0.2 M $NaCO_3$.

Calibration of Fluorimeter

Make a 1 mM stock of MU (Na methyl-umbelliferone Sigma #1508) in MQ water. Dissolve 9.91 mg/50 mls. Wrap in aluminum foil and store at 4 °C. Good for one month.

Make 1 µM and 100 nM solution of MU in 0.2 M Na_2CO_3.
Add 100 µl of 1 mM MU to 900 µl of 0.2 M Na_2CO_3.
1 µM MU: Add 100 µl of this dilution to 9.9 ml of 0.2 M Na_2CO_3.
100 nM MU: Add 1.0 ml of 1 µM MU to 9.0 ml of 0.2 M Na_2CO_3.
Use the 1 µM and 100 nM MU standards to calibrate the fluorimeter.
Set the excitation at 365 nm and emission at 455 nm.

96 well plate assay

1) Pipet up and down several times. Remove 150 µl of sample to a new 96 well plate. Store the new plate at 4 °C. Take original plate that contains approximately 50 µl and use it for the assay.
2) Freeze and thaw one time at -80 °C.
3) Add 150 µl of GUS assay buffer (pipet up and down to mix)
4) Incubate at 37 °C for 2 hours to overnight.

Solutions

GUS assay buffer

Component	Stock	Volume	Concentration
$NaPO_4$ pH 7.0	1 M	2.5 ml	50 mM
2-Mercaptoethanol	14.4 M	35 µl	10 mM
EDTA	0.5 M	1.0 ml	10 mM
Sarcosyl	10 %	0.5 ml	0.1 %
Triton X-100	10 %	0.5 ml	0.1 %
Water			45.47 ml
Total Volume			50 ml

Mix then add 22 mg of 4-Methyl umbelliferyl β-D-glucuronide
Mix and store at room temperature. Good for several months.

Stop Buffer
0.2 M Na_2CO_3 1.06 g/50 ml

Appendix 19: Exonuclease III Deletion Procedure

1) Dissolve 10 µg in 60 µl of 1x Exo III buffer
2) Prepare 12 or 24 tubes (0.5 ml) marked with the appropriate time points and aliquot out 7.5 µl of S_1 mix containing S_1 Nuclease

S_1 mix	27 µl	10x S_1 buffer
	172 µl	water
	pinch of	S_1 nuclease (pinch = < 0.5 µl)

3) Incubate 30 µl (for 12 tubes) or 60 µl (for 24 tubes) of DNA in Exo III buffer at the desired deletion temperature. Deletion rates are approximately 37 °C/300 bp or 30 °C/150 bp
4) Add 2.9 µl (for 30 µl of DNA) or 5.8 ul (for 60 µl of DNA) of Exo III 217 units/µl. Quickly mix and place again at the desired deletion temperature.
5) Remove 2.5 µl at each time point and mix with the appropriate S_1 tube.
6) Incubate S_1 tubes at 30 °C for 20 mins.
7) Add 1 µl of S_1 Stop solution to each tube.
8) Incubate tubes at 70 °C for 10 mins.
9) Add 2 µl of a mixture containing 1 µl of Klenow mix with Klenow at 0.5 units/µl and 1 µl of 0.125 mM dNTP's mix to each tube.
10) Incubate at 37 °C for 5 mins.
11) Total volume of each tube is 13 µl
12) DNA samples may now be run out on a TAE gel to determine the extent of the deletion. Recommend loading 3µl for determining deletion distance and 6-12 µl if samples are to be gel purified. Load the last deletion time point next to the first deletion time point to allow for easier analysis of the deletion distance. Samples loaded on a gel may be run into low melt agarose and cut out for immediate ligation. Running the samples out on a gel and into low melt agarose is strongly recommended when particular deletion size is desired. DNA samples may also be ligated directly without running them on a gel.

Ligation Reaction
10 µl of deletion DNA
10 µl of5X ligase buffer
30 µl of MQ water
1 µl of Ligase 1 unit/µl
Incubate overnight at room temperature

Solutions
10X Exonuclease III buffer
660 mM Tris pH 8.0
6.6 mM $MgCl_2$

10X S_1 buffer

Component	Stock	Volume
KOAc pH 4.6	3 M	1.1 ml
NaCl	5 M	5.0 ml
Glycerol		5.0 ml
$ZnSO_4$		30.0 mg

S1 Stop solution

0.3 M	Trizma Base (no HCl)
0.05 M	EDTA pH 8.0

Klenow mix

Component	Stock	Volume
Tris pH 8.0	0.1 M	3 µl
$MgCl_2$	1.0 M	6 µl
MQ Water		20 µl

Appendix 20: S1 Nuclease Protection

Labeling DNA Probe (3'end labeling)

1) Linearize DNA probe. Verifiy digestion on a gel.
2) In a 1.5 ml eppendorf, set up the following:

DNA: (2 µg)	X µl
10X T4 DNA Polymerase buffer:	2 µl
MQ water:	X µl
	20 µl

3) Incubate at 70 °C for 5 mins, cool on ice 2-3 mins
4) Add 1 µl of T4 DNA Polymerase (1 unit/µl), mix and transfer to 37 °C for 5 mins
5) Add 1 µl of 2 mM (dATP,dTTP,dGTP) stock.
6) Add 2 µl of ^{32}P dCTP (3000 Ci/mmole), mix and incubate 5 mins at 37 °C.
7) Add 1 µl of cold 2 mM dCTP and incubate at 37 °C for 10 mins.
8) Transfer to 70 °C to stop reaction.
9) Dilute reaction to 40 µl and store at -20 °C.

S1 Nuclease Treatment

1) Calculate the quantity of DNA to use for hybridization to RNA by estimating the molar quantity of specific mRNA present in total RNA of infected baculovirus cells. Use a 5 molar excess of DNA probe:specific mRNA. (I recommend using 0.05 ug of labeled probe for 30 µg of total RNA for a baculovirus late gene.)
2) Add the 1 µl of 0.05 µg/µl of labeled DNA probe and 30 µg of total RNA to a 1.5 ml eppendorf tube.
3) Add 10 µl of 3 M NaAc and MQ Water to total volume of 100 µl.
4) Add 200 µl of 95% ETOH mix and store at -80°C for 20 mins.
5) Pellet in microfuge 20 mins. Respin and remove all supernatant.
6) Resuspend pellet in 20 µl of S1 Hybridization buffer(resuspend with pipet to make sure it is resuspended)
7) Incubate at 85 °C for 15 mins.
8) Transfer very quickly to 52 °C water bath (using a beaker with at least 52 °C water.

9) Incubate 3 hours at 52 °C
10) While tubes are still in 52 °C bath, add 300 µl of ice-cold S1 nuclease digestion buffer (with 48,000 units of S1 nuclease per ml). Transfer to ice bath for 2-3 mins.

The proper amount of S1 nuclease needs to be determined by each individual. If the the S1 nuclease is fresh then 600 to 6000 units is probably sufficient.

11) Incubate at 25 °C for 30 mins.
12) Add 75 µl of S1 nuclease termination plus 1 µl of 10 µg/µl of yeast RNA as carrier.
13) Add 2 volumes of ETOH (800 µl), and mix
14) Store at -80 °C for 20 mins and then microfuge for 20 mins.
Resuspend in a mixture of 4 µl of MQ water and 12 µl of loading buffer.

SOLUTIONS
T4 DNA Polymerase Buffer: (45 mM Tris pH 8.0, 5 mM $MgCl_2$, 5 mM DTT)

10X TDPB:	1.0 ml
1 M Tris pH 8.0	450 µl
1 M MgCl2	50 µl
100 mM DTT	500 µl

S1 Nuclease Hybridization Buffer: (80% formamide, 40 mM PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl)
5X Hybridization buffer without formamide 50 mls

PIPES	3.025 g
NaCl	6.845 g
EDTA	0.085 g

pH to 6.4 (PIPES will not fully dissolve until pH 6.4 is reached)
Bring to 50 ml with MQ water. Filter Sterilze and store at 4 °C.

Add 1 volume of 5X hybridization buffer to 4 volumes of 100% deionized formamide. Aliquot in 500 µl and store at -80 °C.

S1 Nuclease Digestion Buffer: (0.28 M NaCl, 0.05 M NaAc pH 4.4, 4.5 mM $ZnCl_2$, 20 µg/ml of denatured Calf Thymus DNA)

50 mls

NaCl	2.9 g
3 M NaAc pH 5.2	1.25 ml
$ZnCl_2$ Fw 136.3	30.7 mg

pH to 4.4 with acetic acid
Bring to 50 ml with MQ water, and Filter Sterilize.
Add 200 µl of denatured Calf Thymus DNA (5 mg/ml).

To denature Calf Thymus DNA should be placed in a boiling water bath for 2 min, then quenched on ice. Aliquot and Store at 4 ^{o}C.

Finally added S1 Nuclease just before use and keep solution on ice.

S1 Nuclease Termination Buffer: (2.5 M NH_4Ac, 50 mM EDTA)

40 ml

7.5 M NH4Ac	13.3 ml
0.5 M EDTA pH 7.4	4.0 ml
MQ water	22.7 ml

Reference: Favoloro et al., 1980. Methods of Enzymology 65:718-749.

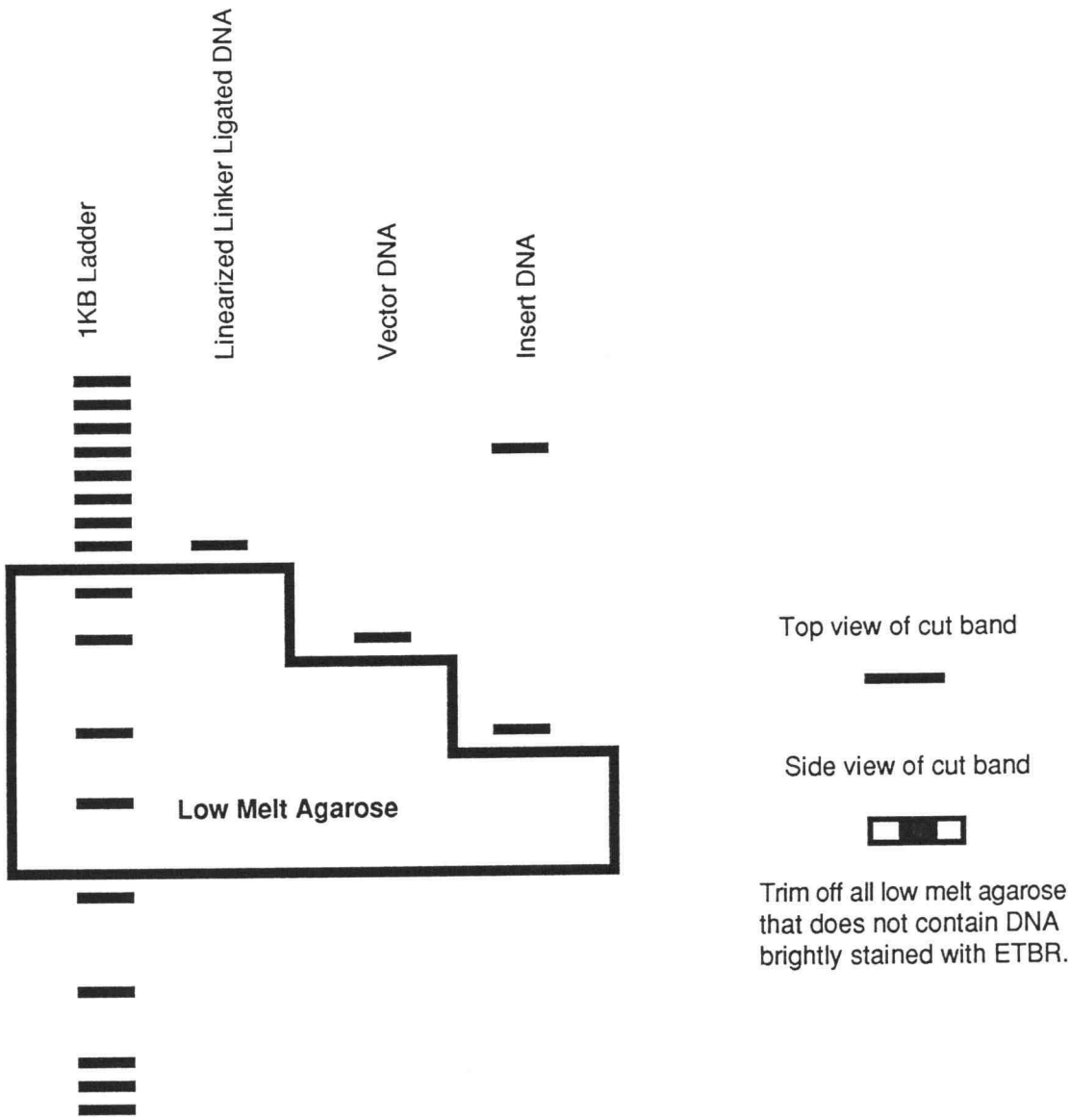
Appendix 21: Low Melt Agarose Cloning and Addition of DNA Linkers

Low Melt Agarose Cloning

1) Load and run out your DNA digestions on a TAE gel to separate desired fragments.

2) Always use only the long UV transilluminator to visulalize your gel

2) After separation of desired fragments is complete cut away the agarose below the desired bands as illustrated on the next page. Place the tray that contains the gel with the removed agarose below your bands in the 4 ^{o}C cold room. Pour the low melt agarose into the hole and let solidify. Use Only SEA PLAQUE LOW MELT AGAROSE from FMC. After solidification, place gel back in electrophoresis box and run desired bands into the low-melt agarose.



With a razor (that is washed between each sample), cut the desired bands from the low-melt agarose. You are only interested in agarose that contains DNA brightly stained with ETBR. Turn the cut-out agarose block of DNA on its side and trim away all agarose not brightly stained with ETBR. Place the block in an eppendorf tube. Note the intensity of the various bands as, this will help you determine what volume of each of the bands should be used in ligation. Store at 4 ^{o}C until needed.

Low-Melt Agarose Ligations

1) Determine on the ratio of insert to vector DNA.

If vector is not cipped, use a molar ratio of 1 to 1 insert/vector.
If vector has been cipped, you may want to use a molar ratio of 1 to 1 insert/vector or 1 to 2 insert/vector. Remember, vector should not be able to religate. Using more vector will prevent insert to insert ligations.

After determining the proper insert to vector ratio, set up the ligation reactions. First, mix MQ water and 5X ligation buffer together then put DNA samples in the 70 ^{o}C water bath to melt the agarose (approximately 5 mins). First mix the DNA-agarose samples to ensure homogeneity. Quickly mix the insert DNA with the water-buffer mixture. Rapidly follow with vector DNA, then with Ligase. Use another pipetman set at 20 µl to give the final mixing by pipeting up and down. Be careful not to generate air bubbles.

Always do a vector control ligation reaction with each vector you use. You may also want to do a insert control ligation if you had a difficulty separating insert from the parent vector.

Ligation Reaction	
MQ water	X µl
5X ligation buffer	4 µl
Insert DNA	X µl
Vector DNA	X µl
Ligase (1 unit/ul)	1.5 µl
Total volume	20.0µl

Vector Control Ligation Reaction		
MQ water	X µl	X= MQ water + insert from ligation reaction
5X ligation buffer	4 µl	
Vector DNA	X µl	same amount as in ligation reaction
Ligase (1 unit/ul)	1.5 µl	
Total volume	20 µl	

Store ligations for 2 hours to overnight at room temperature and then transform.

Kinase DNA Linkers

Linkers 0.5 µg/µl	1.5 µl
1 M Tris pH 8.0	3.0 µl
0.2 M $MgCl_2$	1.5 µl
0.1 M DTT	1.5 µl
10 mM ATP	1.3 µl
MQ water	21.2 µl
	30.0 µl

Add 5 units of T4 polynucleotide kinase (5 units/µl)
Incubate for 45 mins at 37 °C
Freeze at -20 °C or use immediately

Blunt 5' DNA overhangs using Klenow or T4 DNA polymerase
Klenow (preferred over T4 DNA polymerase)

DNA (0.1 µg/µl)	18 µl	
10 mM dNTP's	4 µl	(1 µl of each)
10X Nick translation buffer	3 µl	
MQ water	5 µl	
Total	30 µl	

Add Klenow (5units/µl) 1 µl
Incubate at 37 °C for 30 mins
Heat inactivate at 65-70 °C for 10 mins

10X Nick translation buffer: (5 mls)

0.5 M Tris pH 7.2	2.5 mls of 1 M Tris pH 7.2
0.1M $MgSO_4$	0.5 mls of 1 M $MgSO_4$
1 mM DTT	50 µl of 0.1 M DTT
MQ water	1.95 mls
500 µg/ml of BSA fraction V	2.5 mg

Divide into small aliquots and store at -20 °C

T4 DNA polymerase (may also be used to blunt 3' overhangs)

DNA (0.1 µg/µl)	18 µl	
10 mM dNTP's	4 µl	(1 µl of each)
New England Biolabs buffer #4	3 µl	
MQ water	5 µl	
Total	30 µl	

For 5' overhangs incubate at 37 °C for 30 mins
For 3' overhangs incubate at 12 °C for 15 mins
Heat inactivate at 65-70 °C for 10 mins

Note: Using T4 DNA polymerase on 3' overhang leave out the dNTP's for first 5 mins of incubation (this facilitates the removal of the 3' overhang).

S1 Nuclease treatment to blunt 3' overhangs (preferred method)

1) Digest 3 µg of DNA with the appropriate restriction enzyme in a 30 µl reaction. Confirm linearization of the plasmid on a agarose gel.
2) Add 25 µl of S1 mix to the 30 µl of DNA.

S1 MIX

27 µl	of 10X S1 buffer
172 µl	of MQ water
0.25 µl to 0.5 µl	of S1 nuclease

3) Incubate at 30 °C for 20 mins.
4) Add 1 µl of S1 stop solution
5) Incubate tubes at 70 °C for 10 mins.
6) Add 30 µl of Klenow mix and 0.5 µl of Klenow (5 units/µl). Transfer to 37 °C.
7) Incubate for 3 min and follow with 1 µl of 0.125 M dNTP's stock. Then incubate for 5 mins at 37 °C.
8) If DNA is to be used in linker ligation, extract one time with phenol/chloroform. ETOH precipitate with 50 µl 7.5 M NH_4Ac and 300 µl ETOH 95%. Leave at -80 °C for 15 mins and centrifuge for 20 mins at 4 °C. Remove supernatant. Respin briefly and remove remaining

supernatant. Resuspend in 50 µl of MQ water and use 30 µl in the linker ligation described below.

If DNA is simply to be ligated to itself, then take:

10 µl	of S1 treated DNA mixture
10 µl	of 5X ligase buffer
30 µl	of MQ water
1 µl	of Ligase (1 unit/µl)

Incubate for 2 hours to overnight at room temperature and then transform.

Linker Ligation

Kinased linkers	30 µl
Blunted DNA	30 µl
5X Ligase buffer	17 µl
Ligase (1unit/µl)	8 µl
Total	85 µl

Incubate overnight at room temperature

If the restriction site where the linker was ligated to was already blunt from a blunt end cutter like Sma I then just take 10 µl of the 85 µl ligation and transform. Screen 12 colonies and more than likely, most will have a linker(s) inserted. Beware that these clones may have more than one linker inserted. In most cases you will be inserting something into the newly created linker site. When you reopen the linker site to insert a fragment, digest with excess enzyme to removed extra linkers.

If the restriction site required blunting then I recomend the following:

1) Add 5 µg of tRNA as a carrier and bring the ligation to a volume of 100 µl with MQ water.

2) Add 50 µl of phenol/chloroform and vortex. Centrifuge for 3 mins to set phases.

3) Transfer supernatant to a fresh 0.5 µl eppendorf tube and add 50 µl of 7.5 M NH_4Ac and 300 µl of 95% ETOH. Incubate at -80 °C for 30 mins. Centrifuge at 4 °C for 30 mins. Carefully remove supernatant and recentrifuge for 5 mins. Remove the remaining supernatant with a sequencing tip.

4) Resuspend DNA pellet in 81 µl of MQ water. Add 10 µl of the appropriate 10X restriction buffer and 9 µl of the linker restriction enzyme (10 units/µl). Incubate for 5 to 6 hours.

5) Add 5 µg of tRNA as a carrier, 50 µl of 7.5 M NH_4Ac and 300 µl of 95% ETOH. Incubate at -80 °C for 30 mins. Centrifuge at 4 °C for 30 mins. Carefully remove supernatant and recentrifuge for 5 mins. Remove the remaining supernatant with a sequencing tip.

Resuspend in 20 µl of MQ water and mix with 5 µl of loading buffer. Load and run on a TAE gel. When the linearized DNA band has been resolved from the linkers, cut a hole in the agarose below the band and replace with low-melt agarose (see above).

Appendix 22: Gene Clean DNA Fragments

Glassmilk powder Concentration = 0.5 mg glass/µl
Binding capacity: 1 mg glass binds ≈ 2 µg DNA

Method:

1. Bring the volume of DNA in solution up to 200 µl with H_2O.
   For gel purification, excise the band from the gel.
2. Add 2-3 volumes of NaI solution and MIX.
   For gel purification, place the eppendorf tube at 55° C to melt the agarose (app. 5-10 min.)
3. Add 5 µl of GLASS, VORTEX.
4. Place on ice for 5 mins.
5. Burst spin for 5 sec
6. Discard the NaI solution
7. Resuspend 3x with "NEW" Buffer
   Add 400 µl of NEW buffer each time and vortex briefly
   Burst spin and remove the NEW buffer
8. After the last spin, burst the tube again to remove residual buffer
9. Elute the DNA from the glass
   Add X µl of H_2O or 0.1x TE (X≥ 5 µl) MIX
   Incubate at 55° C for ≈5 minutes
10. Burst spin
11. Remove supernatant containing the DNA. Be sure not to get any glassmilk with the supernatant.
12. If desired, the elution from the glass may be repeated.

<u>NaI Solution</u>
Saturated solution of NaI (Refractive index of 1.5000) with sodium sulfite added as an antioxidant.

In a 70° C waterbath
Add 2 g Na_2SO_3 (sodium sulfite) to 100 ml MQ H_2O and dissolve.
Add 180 g NaI to the 100 ml
Add ≈1/4 the amount of NaI at a time until all is dissolved (note: the last bit may not go completely into solution—the solution may remain cloudy)
Let sit overnight at room temperature wrapped in foil.
Filter through 0.45 µ nitrocellulose to remove undissolved NaI
Store @ 4° C in the DARK

<u>Glass powder</u>:
1 g of glass powder in 2 ml of H_2O for stock (0.5 mg/ml)
see Vogelstein and Gillespie for preparation of glass or

BIO 101 Inc. , P.O. Box 2284, La Jolla, CA 92038-2284
1-800-424-6101
Product = "GENECLEAN"

NEW Buffer

NEW buffer = 50% (20 mM Tris-HCl [7.2], 0.2 M NaCl, 2 mM EDTA)
50% EtOH

References:

Vogelstein B. and D. Gillespie 1979. Preparative and analytical purification of DNA from agarose. PNAS 76(2): 615-619.

Struhl, K. 1985. BioTechniques 3: 452.