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Baculovirus nucleocapsid aggregation (MNPV vs SNPV): an evolutionary strategy, or a product of replication conditions?

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Abstract Lepidopteran nucleopolyhedroviruses are members of the *Baculoviridae* and have been categorized as having two morphotypes of occluded virions: multiple nucleocapsids or single nucleocapsids within the virion envelope. Although it is a definitive characteristic of specific viruses, it appears to lack a defined genetic basis and is independent of virus phylogeny. This review summarizes the factors that appear to influence this trait and the role that it may play in virus biology.

Keywords Baculovirus · Virion morphology · Virion aggregates · *Alphabaculovirus* · Lepidopteran baculovirus

The *Baculoviridae*, a large family of viruses with double-stranded DNA genomes of 80–180 kbp, appear to have undergone host-dependent evolution and are divided into four genera based on the three orders of insects they infect and phylogenetic analyses. These include the *Alpha-* and *Beta-* (*Lepidoptera*), *Gamma-* (*Hymenoptera*), and *Deltabaculoviruses* (*Diptera*). The two lepidopteran genera are closely related and comprised nucleopolyhedroviruses (NPVs) that have multiple virions occluded per inclusion body (*Alphabaculoviruses*) and the granuloviruses (GVs) (*Betabaculoviruses*) that normally have a single virion per smaller occlusion body (Fig. 1). In addition, the lepidopteran NPVs are divided into Group I and Group II. The Group I viruses form a distinct lineage and contain a suite of about 12 genes not found in Group II, the most prominent of which encodes the envelope fusion protein GP64. The Group II viruses are much more

diverse than Group I, but all lack GP64 and appear to utilize a different fusion protein called F. Because of their diversity, it is unclear whether Group II viruses form a single lineage. It is thought that the Group I viruses obtained *gp64* in a recombination event, in which it displaced the fusion function of F and this led to the development of that lineage [1–3]. However, the Group I viruses retained an ortholog of the F fusion protein (e.g., *Ac23* in *Autographa californica* MNPV (AcMNPV)), but it does not independently induce fusion and its role in virus infection is unclear. Finally, like the lepidopteran Group II and GV, the dipteran viruses encode an ortholog of the F fusion protein, but no GP64 protein, whereas the hymenopteran baculoviruses encode no proteins related to either of the known fusion proteins.

Nucleocapsid aggregation: multiple versus single nucleocapsids (MNPV vs SNPV)

A prominent feature of different types of lepidopteran NPVs is the organization of nucleocapsids within polyhedra, the inclusion body, into either single or aggregates of multiple nucleocapsids within an envelope (Fig. 1a). For example, in some NPVs, there can be from 1 to 15 nucleocapsids per envelope, with bundles of 5–15 predominating. In contrast, strains defined as having a single nucleocapsid per envelope rarely show more than one nucleocapsid per envelope [4–6]. Because this feature is so distinctive and characteristic of specific isolates, it was incorporated into the early nomenclature such that NPVs were categorized as either multiple (MNPV) or single (SNPVs) (also previously called multiple or singly embedded virions (MEV and SEV)). In addition, whereas MNPVs and SNPVs are both found in lepidopteran viruses,

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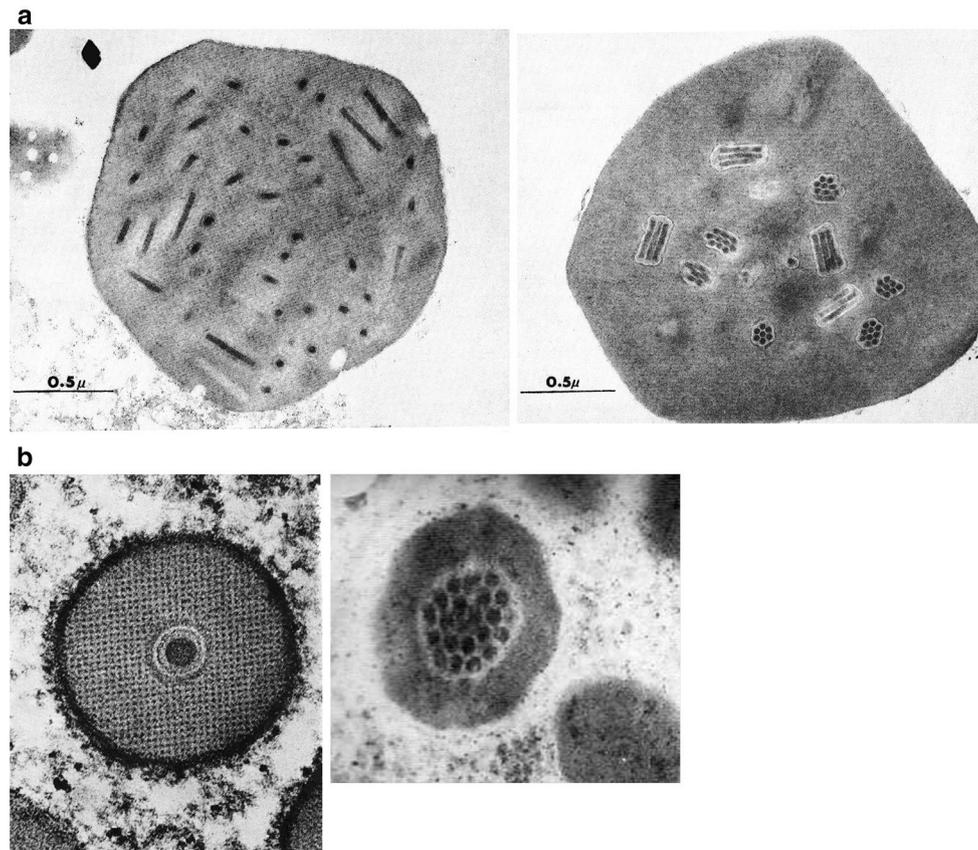


Fig. 1 Baculovirus Morphotypes. **a** Two nucleopolyhedroviruses pathogenic for *O. pseudotsugata* showing single (OpSNPV) (Left panel) and multiple (OpMNPV) (right panel) nucleocapsids per envelope. Reprinted from Hughes and Addison [4] with permission from Elsevier. **b** Granulovirus morphotypes. Left panel: Cross section

of a granulovirus of *Plodia interpunctella*. Right panel: Multiple granulovirus occluded nucleocapsids of *Cydia pomonella*. Reprinted from Arnott and Smith [44] and Falcon and Hess [7], respectively, with permission from Elsevier

only SNPVs are observed in the dipteran and hymenopteran viruses. GVs are also categorized as singly enveloped; however, multiple GVs have been described in several reports, reviewed in [7] (Fig. 1b).

An MNPV and a SNPV of *Orgyia pseudotsugata* (Fig. 1a) were intensively investigated because they were present as a mixture in the original preparations of the viral insecticide TM biocontrol-1 and were found to have distinctive properties. For example, compared to OpMNPV, OpSNPV has fewer nucleocapsids per polyhedron (670 vs 1600) [8, 9], has longer nucleocapsids (277 vs 227 nm) [4], a larger genome [10] with a lower G + C content [11], was thermo inactivated at a lower temperature (55 vs 60 °C), and causes a significantly longer mean survival time (6.85 vs 5.83 days) [12]. This could result in relatively more OpSNPV production because the insects can continue to feed and grow right up until they die. In addition, whereas OpSNPV is widely distributed throughout the western USA and British Columbia (BC), OpMNPV is found in the northern part of this range starting in northern Oregon and

extending into BC where it predominates [4, 13, 14]. In competition studies in which larvae were fed various combinations of the two viruses, OpMNPV out competed OpSNPV and most of the insects appeared to be exclusively infected with OpMNPV with the remainder predominately containing mixtures of both viruses [8]. Since the viruses differ significantly in gene content and diversity being members of Group I (OpMNPV) [15] and Group II (OpSNPV) [16], it is not possible to attribute these differences specifically to the MNPV or SNPV morphotype. Similar comparisons have also been reported on *Heliothis zea* SNPV (HzSNPV and AcMNPV) [17].

With the accumulation of DNA sequence data that allowed for the determination of definitive phylogenetic relationships, it was found that the MNPV and SNPV division did not conform to the phylogeny of the viruses. For example, at one point, the *Bombyx mori* NPV (BmNPV) was considered the type virus for SNPVs because of its production of predominantly single nucleocapsids [18]. However, sequence data indicate that

BmNPV is closely related to AcMNPV (both belong to Group I), whereas other MNPVs such as *Lymantria dispar* (LdMNPV) and *Spodoptera exigua* (SeMNPV) are more distantly related Group II viruses, which also include SNPV-type viruses.

Despite agreement that the MNPV and SNPV designation is not a useful phylogenetic trait, it continues to be employed, in part for historical continuity, and also because it can be a convenient way to distinguish different viruses that are pathogenic for the same host, e.g., OpMNPV and OpSNPV which both infect *O. pseudotsugata*, (Fig. 1a) (see above).

Are there genetic determinants of the MNPV morphotype?

Although the M or SNPV morphotype does not consistently reflect viral phylogeny, several genes have been identified that influence the production of MNPVs. It was found that AcMNPV deleted for *ac23*, an ortholog of the F fusion protein gene of Group II viruses (see above), shows an elevated percentage of singly enveloped virions, 45 % vs 11–22 % for different virus constructs that contained *ac23* [19]. Although characterized as a BV-associated protein in AcMNPV [20], OpMNPV [21], and HearNPV [22], Ac23 was reported to be associated with AcMNPV ODV [23], suggesting that it could be directly involved in formation of the MNPV morphotype. Also a knockout (KO) of *ac43* appeared to increase the number of SNPVs in occlusion bodies, and co-infection of the *ac43* KO virus with a virus encoding *ac43* restored the predominance of the MNPV morphotype [24]. It has also been shown that deletion of *ac92*, a core gene encoding a sulfhydryl oxidase, results in virions that resemble the SNPV morphotype [25]. However, they are not infectious, indicating that they have a structural defect in one or more viral or possibly cellular proteins required for the production of viable virions. When an ortholog of *ac92* (*tn79*) from an SNPV (*Trichoplusia ni* SNPV) was inserted into AcMNPV deleted for *ac92*, the infectivity of the virus was rescued. Although viral titers were reduced by tenfold or more depending on the multiplicity of infection (moi) used and the cell line infected, the virus appeared to have the MNPV morphotype restored [26]. These data indicated that an SNPV sulfhydryl oxidase did not independently specify the SNPV morphotype. In an investigation on drug resistance in DNA polymerase, it was observed that point mutations in domains II or III of the AcMNPV DNA polymerase gene caused a major change in the ratios of S to MNPV types [27]. For example, wt AcMNPV showed only about 3 % SNPV virions in the ring zone of infected cell nuclei, whereas a point mutant in domain III or a double mutant in

domains II and III resulted in 61 and 89 % singly enveloped virions, respectively. Similar elevated proportions of SNPVs were also observed in polyhedra produced by these mutants. The production of infectious budded virus (BV) and genomic DNA was also reduced in the mutants. Therefore, the expression of some baculovirus genes can influence MNPV production and this may be linked to the efficiency of virion replication or virion integrity.

It also appears that the type of cell infected can influence the morphotype. In a report describing the isolation of a virus pathogenic for *Bombyx mandarina* (BomaNPV S2) (a close relative of BmNPV), it was observed that the virus grew in both *B. mori* 5 (Bm5) and *T. ni* cells. In Bm5 cells, the virus was present as an SNPV. However, in *T. ni* cells, it had an MNPV morphotype [28]. Therefore, the viral morphotype was influenced by the physiology of the cell, or how the cell responds to viral infection, or cell specific factors. Similar possibilities were summarized in the report by Falcon and Hess [7] who suggested that the presence of multiple nucleocapsids in *Cydia pomonella* GV-infected cells (Fig. 1b) may have been due to cellular factors or physiological conditions.

Are the budded virions of MNPVs present as singly enveloped nucleocapsids?

It is generally assumed that the budded virus (BV) of AcMNPV is present as singly enveloped nucleocapsids. This is evident in many pictures taken of BV preparations, e.g., see, [29, 30]. It has also been shown that in infected insects, BV comprised more than a single nucleocapsid may be present. For example, in studies of AcMNPV within 4 h after *T. ni* larvae were fed $0.5\text{--}1.8 \times 10^7$ occlusion bodies, there appears to be concentrations of nucleocapsids abutting and apparently budding through the basal lamina of midgut cells. It was reported that both single nucleocapsids and aggregates were observed in the hemocoel adjacent to the basal lamina [31]. Similarly, reports of *Helicoverpa zea* (HzSNPV) show groups of nucleocapsids apparently budding through the plasma membrane [32]. It is not clear if these are newly replicated or nucleocapsids that passed through the cell without replicating (see below). However, under normal circumstances, infections initiated by both S- and MNPV-type viruses pass through a single nucleocapsid phase, e.g., [29, 30], in the form of BV that spreads the viral infection within the insect. It is not until later in infection when nuclear envelopment and occlusion of nucleocapsids are initiated that in some lineages, the MNPV type predominates in the nuclei of infected cells. These MNPVs are destined to become embedded into occlusion bodies.

Consequently, it appears that all baculoviruses may be present predominantly as single nucleocapsids in the form of BV, and the appearance of MNPVs appears later in infection prior to or concomitant with occlusion.

Production of the S and MNPV morphotypes

Whereas one can readily envision a single BV nucleocapsid converting from the budded phase to the SNPV-occluded phase, the conversion of a BV to an occluded MNPV would be more complex involving both nucleocapsid aggregation and the envelopment of multiple nucleocapsids. *Alphabaculoviruses* develop in the virogenic stroma in association with concentrations of microvesicles from which it is thought that the envelope of occluded virions is derived [33] (Fig. 2). MNPV nucleocapsids have often been documented, aligned with a membrane-like structure (Fig. 2). It is thought that the MNPV envelope may be derived from this structure and, because of the association with multiple nucleocapsids, may yield the MNPV morphotype. In contrast, the SNPV nucleocapsids, although often highly concentrated, appear to be enveloped individually (Fig. 2). Many virion-associated proteins such as the tegument protein (GP41), nucleocapsid proteins (VP39 and Ac98 (38 K)), and envelope proteins (ODV-E25 and ODV-E66) may be capable of interacting with a variety of other virion proteins [34]. In MNPVs, features of these proteins could facilitate nucleocapsid aggregation and interaction with the ODV envelope. Mutations in MNPVs that reduce the concentration of nucleocapsids or interfere with inter-nucleocapsid interactions or their association with the envelope precursor structure might cause the production of SNPV-like virions.

Is there an evolutionary advantage to the MNPV morphotype?

Two theories have been presented to explain the possible advantage of the MNPV morphotype.

- i. First, it has been suggested that the MNPV format allows the infection of single cells by multiple nucleocapsids and this could serve to repair damaged DNA via recombination [35]. Therefore, if aggregated nucleocapsids from MNPVs coinfect cells simultaneously, fatally damaged genomes could recombine to produce a viable genome. Whereas there is no experimental evidence to support this theory, it is attractive because baculoviruses are highly sensitive to UV radiation and highly

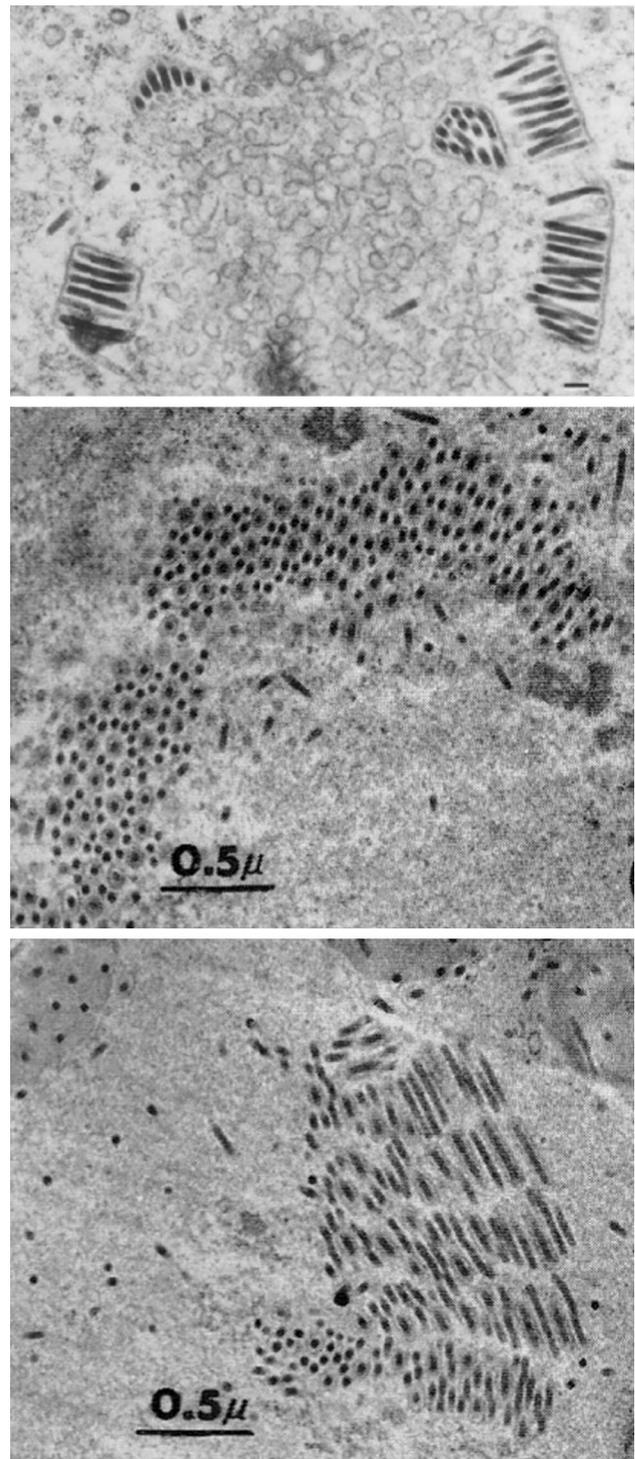


Fig. 2 Development of M- and SNPVs. *Top panel:* AcMNPV virion development in area of microvesicles. *Middle and bottom panels:* OpSNPV development. Reprinted from Fraser [33] and Hughes and Addison [4], respectively, with permission from Elsevier

recombinogenic, and this would provide a mechanism for them to retain or prolong their infectivity in the environment.

ii. Second, it has been proposed that under some circumstances, MNPV-derived nucleocapsids can transit through gut cells, bypassing replication, and bud directly into the hemolymph [31], or tracheal cells [36]. This has also been observed in cultured *T. ni* cells infected at high moi (200) of AcMNPV; many virions appear to become associated with the plasma membrane from within the cell 2–4 h postinfection suggesting that they were not newly replicated virions but from the original inoculum [37]. It is thought that this ‘pass-through’ phenomenon may accelerate the ability of the virus to establish the infection because if multiple nucleocapsids infect a cell simultaneously, some could enter the nuclei, begin replication, and prepare the membrane for viral budding by expressing their fusion protein GP64, whereas others could pass directly through the cell, bud through the virally modified membrane, and spread the infection elsewhere. This may be a mechanism for accelerating systemic infection and avoiding the necessity to undergo a complete cycle of replication in gut cells as the insects appear to have developed a method to combat the virus by eliminating infected midgut cells in a process called sloughing in which the infected cells detach from the gut lining and die.

The comparative ability of MNPV and SNPV to establish secondary infections was examined for an AcMNPV construct expressing *lacZ* [36] in which the two morphotypes were enriched using sucrose gradient centrifugation. This resulted in an SNPV fraction that was 86 % single and 14 % with 2–4 nucleocapsids and an MNPV fraction that was 78 % 2–4 and 22 % 5 or more nucleocapsids per envelope. The subsequent comparisons were based on using an LD70 rather than the number of nucleocapsids. The SNPV preparation contained eightfold more protein and 39 % more genome copies comprising 4.5 more virions than the MNPV fraction. Since the assay was based on an LD70, this suggested that the MNPV was more infectious per enveloped unit than the SNPV preparation. Although the SNPV inoculum produced more infected foci at 6 hpi, they were predominantly located in midgut cells, whereas the MNPV foci were multicellular and had spread to tracheal cells, indicative of a systemic infection. The authors concluded that the MNPV morphotype accelerated secondary infection of tracheal cells and suggested that there was competition between viral midgut cell infection and the sloughing of these cells by the insect. Although the SNPV fraction established more primary midgut cell infections, the MNPVs were more successful in mitigating the effects of sloughing by more rapidly establishing systemic infection. In cultured *T. ni* cells, AcMNPV BV is not detected until after 10 hpi [38] in contrast to tracheal infections observed by 6 hpi with the MNPV

inoculum in larvae. If the replication time is similar in cultured cells and larvae, it would suggest that MNPV were able to produce secondary infections several hours before the virus would normally take to replicate, suggesting that some viruses had passed through cells and established the secondary infection. Another report [39] compared the infection of *Heliothis virescens* larvae with AcMNPV and *H. zea* SNPV (HzSNPV). Although primary infection of midgut cells appeared earlier with HzSNPV, secondary infections occurred at about the same time for both viruses, suggesting a shorter delay between primary and secondary infections for AcMNPV. However, interpretation of these results is complicated by the fact that there are major differences in the genome content between the viruses and consequently it is not possible to attribute the results of infection to the S or MNPV morphotype. For example, AcMNPV is a member of Group I, whereas HzSNPV is a member of Group II *Alphabaculovirus*.

The two different *Alphabaculovirus* envelope fusion proteins (GP64 and F) have both early and late promoters suggesting that part of the common infection strategy of both S and MNPVs is to prepare the cell membrane for nucleocapsid budding early and then maintain it throughout the infection. This may have been the original impetus for this type of regulation because it would likely accelerate the infection irregardless of the M- or SNPV morphotype. The ‘pass-through’ theory is dependent upon the early expression of GP64 because it could allow some MNPV-derived nucleocapsids to directly pass through the cell and bud through the GP64-modified cell membrane without undergoing replication. Comparison of the time course of infections of AcMNPV expressing *lacZ* with the early *gp64* promoter deleted has been examined in 4th instar *H. virescens*, *T. ni*, and *S. exigua* larvae. Lack of early *gp64* expression caused a delay in secondary infection of larvae, suggesting that the MNPV morphotype facilitates the pass-through and establishment of secondary infection that may be an advantage in accelerating the secondary infection, thereby counteracting the ability of insect larvae to slough infected midgut cells. However, this phenomenon appeared to vary with the host insect and the specific stage of the development of the larvae. For example, deletion of the early promoter had no effect on infection of AcMNPV in newly molted 4th instar *S. exigua* larvae; however, an effect was observed at 16 h post 4th instar molt in *S. exigua* [40, 41]. Therefore, the advantage of early expression of *gp64* appears to be dependent on both the type of insect infected and the developmental stage of the insect.

The ‘pass-through’ theory may only be applicable to the MNPV type of virus, because it is dependent upon a cluster of connected nucleocapsids simultaneously infecting single cells, after which they would separate in the cytoplasm

with some entering the nucleus to undergo conventional replication. This theory is complicated by a lack of understanding as to what happens to the aggregated MNPV nucleocapsids when they are released from polyhedra in the harsh environment of the insect midgut where they are exposed not only to alkaline pH, but also to a variety of enzymatic processes. It is not clear whether most MNPV nucleocapsids would remain associated under these conditions and bind to and enter cells as aggregates, or be separated from one another by the environment of the midgut and enter cells as single nucleocapsids with partial or fragmented envelopes. However, in studies of Ac-MNPV, within 4 h after *T. ni* larvae were fed $0.5\text{--}1.8 \times 10^7$ occlusion bodies, aggregates of multiple nucleocapsids were observed within the microvilli of midgut epithelial cells. In addition, it was reported that these aggregates separated into single nucleocapsids upon exiting the microvilli into the cytoplasm [31].

As described above, a similar combination of early and late promoters is also present upstream of almost all of the F genes of Group II viruses, suggesting these two types of envelope fusion proteins (F and GP64) may be regulated in a similar manner [42]. Both early and late expression of the envelope fusion protein would ensure that the cell membrane of the infected cells could be modified to allow the budding of nucleocapsids destined to become BV at both early and later times postinfection. However, for SNPVs, it is difficult to link the regulation of the fusion protein to the possible pass-through transit of nucleocapsids, because it would require extremely high levels of virus inoculum to ensure infection of single cells with more than one virion so that both nucleocapsid pass through and replication with fusion protein synthesis could occur in the same cell. However, under certain circumstances, for example during viral infections of dense populations of insects, vast numbers of virions could be consumed by single insects. The advantage to the virus of accelerating the infection by a few hours under these conditions is unclear unless the sloughing of infected midgut cells is so efficient that it could block the infection.

In contrast to the regulatory region of the F gene in Group II viruses, the regulatory region of the F gene orthologs in nine sequenced granulovirus genomes examined lack conventional early promoter consensus sequences (TATA + CAGT) in the proper context, and only three had late promoter elements within 200 nt upstream of the ATG [43].

Is there an evolutionary advantage for the SNPV morphotype?

If all things are equal in an SNPV vs MPNV infection in terms of the number of genomes replicated and packaged

into complete nucleocapsids, then the SNPV morphotype may have a clear advantage over the MNPVs because it has the potential to provide many more individual infectious units; and consequently, the probability of contacting a cell and initiating an infection would have a greater probability with SNPVs. This could be a major advantage in situations in which midgut sloughing is delayed or does not occur, or early after virus production before viral genomes have been inactivated by UV light or other phenomena.

Conclusions

As is evident in this review, the significance of the MNPV morphotype is complex. So far a specific causal genetic component has not been identified and although it occurs in most Group I *Alphabaculoviruses*, MNPVs are also common in Group II viruses and sometimes are found in the *Betabaculovirus* (GVs). Furthermore, MNPV formation can be influenced by the type of cell infected, suggesting an environmental or physiological influence. Whereas it may provide an advantage in the establishment of secondary infections, this can be influenced by both the host insect species and the larval stage which further implicates the cell environment. If there was a simple gene-based selective advantage for the MNPV morphotype, one might predict that it would spread throughout the lepidopteran NPVs. However, because of its evident complexity, a genetic basis would require the selection of a complex of interacting phenomena that account for the physiology of the cell and the proper expression of a variety of different viral genes.

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