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Baculovirus

Gene Expression

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Additional information is available at the end of the chapter

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1. Introduction

Baculoviridae is a diverse family of insect viruses with large, double-stranded, circular DNA genomes packaged in rod-shaped, enveloped nucleocapsids. A characteristic feature of baculoviruses is the production of paracrystalline occlusion bodies (OBs) which surround the assembled virions at late times of infection. Baculoviruses produce lethal infections in their hosts and OBs protect the virions in the environment after death of the insect until uptake by another susceptible host. Other insect viruses also produce OBs such as entomopoxviruses (EPV) and cytoplasmic polyhedrosis viruses (CPV). EPV and CPV replicate in the cytoplasm of infected cells; in contrast, baculoviruses replicate within the nucleus. According to the size and shape of OBs, baculoviruses were traditionally classified into two genera: nucleopolyhedrovirus (NPV), which produce large OBs known as polyhedra, and granulovirus (GV), which produce small ovoid OBs or granules. The major proteins that form each class of OB are known as polyhedrin and granulin, respectively. Recently, after several baculoviral genomes have been sequenced, a new classification based on the phylogenetic relationships between species within the family was accepted [1-2]. Four genera were defined: *Alphabaculovirus* (lepidopteran NPV), *Betabaculovirus* (lepidopteran GV), *Gammabaculovirus* (hymenopteran NPV) and *Deltabaculovirus* (dipteran NPV). Baculoviruses are not infectious to vertebrates and they were known from far before they were recognized as viral entities as they produced disease outbreaks in the silkworm rearing. There are more than 600 species described in the literature [3] and some of them are widely used as bioinsecticides to control insect pests in agriculture and forestry [4]. A

feature that differentiates baculovirus from other DNA viruses that replicate in the nuclei of infected cells is that they encode a novel DNA-dependent RNA polymerase. This enzyme, of uncertain evolutionary origin, is responsible for transcription of baculovirus late and very late genes. At this time of the infection the transcription of most cellular genes is shutoff and the synthesis of the polyhedrin/granulin becomes prominent to finally account for up to 95% of total cellular protein production. This high capacity of protein synthesis has been exploited for the development of baculoviruses as vectors for expression of foreign proteins.

2. Two types of virus progeny serve at different steps of host invasion

Alphabaculoviruses and betabaculoviruses produce virions of two phenotypic classes: occlusion-derived virus (ODV) and budded-virus (BV). Nucleocapsids of both types of virus particles are assembled in the nucleus of the infected cell. During the late phase of infection, when proteins of the nucleocapsid are expressed, BVs are produced as the newly formed nucleocapsids exit the cell, acquiring their envelopes from the cell membrane during the budding process. BVs disseminate the infection within the host by entering other cells via a mechanism of receptor-mediated endocytosis. ODVs arise at very late times, when nucleocapsids are enveloped in membrane units derived from the nuclear envelope to finally be embedded in the OBs. OBs persist in the environment after liquefaction of the insect cadaver and are responsible for the horizontal transmission of the virus between hosts. Upon ingestion by a susceptible insect larva, OBs are dissolved in the alkaline environment of the midgut and ODVs are released. The ODVs move through the peritrophic membrane and nucleocapsids are delivered into midgut epithelial cells through a mechanism of membrane fusion mediated by specific viral proteins known as *per os* infectivity factors (PIFs). This primary infection in the midgut is followed by a secondary infection, consisting in the dissemination of BVs to other tissues. In contrast to this infection cycle, which is typical of lepidopteran-specific baculoviruses, infections of gammabaculoviruses and deltabaculoviruses are restricted to the midgut of their hosts.

3. Nuclear events associated with infection

Baculovirus infection causes cells to enlarge and stop dividing; the nucleus swells and forms the virogenic stroma (VS), which is the nuclear compartment where the viral DNA is replicated and the nucleocapsids of virus progeny are assembled. The host chromatin adopts a marginal distribution at 24 hours post infection (hpi), when the VS becomes evident. At this time, it was shown that histone H4 fused to fluorescent protein markers colocalizes with the chromatin in the periphery but not with the VS [5]. The new distribution of the chromatin is determined by the replication of the virus and may have effects

in changes operated in the expression of host genes and the progression of the cell cycle. At late times, ODVs become occluded into OBs in the periphery of the VS.

Apparently, the nucleocapsids of NPVs enter the nucleus through the nuclear pores, whereas the genome of GV is probably injected [6]. Virions of baculoviruses are devoid of histones, in turn the DNA in the nucleocapsid is packed in association with viral protein P6.9, a basic DNA-binding protein. This small polypeptide is rich in arginine, serine and threonine residues, a feature similar to proteins called protamines present in the nuclei of spermatids in many animals and plants. The positive arginine residues in protamines neutralize the negative charges in the DNA backbone while serine and threonine mediate interaction between protamine molecules, resulting in a high condensation of genomic DNA. Once it is uncoated into the nucleus, the DNA dissociates from P6.9 through the phosphorylation of the protein. During infection the viral DNA appears to be organized in the form of nucleosomal-like structures in association with P6.9, as suggested by experiments of micrococcal nuclease digestion of isolated nuclei [7].

4. Transcription program of baculovirus genes

In general, genes of DNA viruses are transcribed in a temporal sequence and the process is highly regulated by infection-derived mechanisms and proteins from both host and viral origin. This stepwise mode of gene expression ensures the availability of gene products required for the progression into the next phase of the infection. Baculoviruses express their genes in three successive phases designated as early, late and very late (figure 1). Early genes are transcribed by the host RNA polymerase II before virus DNA replication, while late and very late genes are transcribed by a virus-encoded RNA polymerase, after starting of viral DNA replication. Products of a number of early genes are required for virus DNA synthesis and for expression of late/very late genes, and at least one late gene product is also needed for expression of very late genes. The progression of the infection into the late phase correlates with the transcriptional shutoff of cellular and early viral genes. Genes belonging to the different temporal classes are encoded in both DNA strands without any associative distribution in the genome. A number of baculovirus genes contains promoters with sequence elements characteristic of both early and late classes. Their transcription is regulated independently at each temporal phase which ensures their expression throughout the infection [8]. Most of our knowledge of baculovirus gene regulation comes from studies in *Autographa californica* nucleopolyhedrovirus (AcMNPV), which is the type species of the family. This alphabaculovirus has a wide host range and causes productive infections in permissive insect cell-lines Sf21 (and its clonal isolate Sf9), derived from *Spodoptera frugiperda* [9] and TN368, from *Trichoplusia ni* [10]. The genome of AcMNPV encodes 154 predicted open reading frames (ORFs) [11]. Roughly, one half corresponds to late genes, according to the sequence elements present in their promoters. About 25 gene products, mostly of early genes, have functions directly or indirectly related to gene expression.

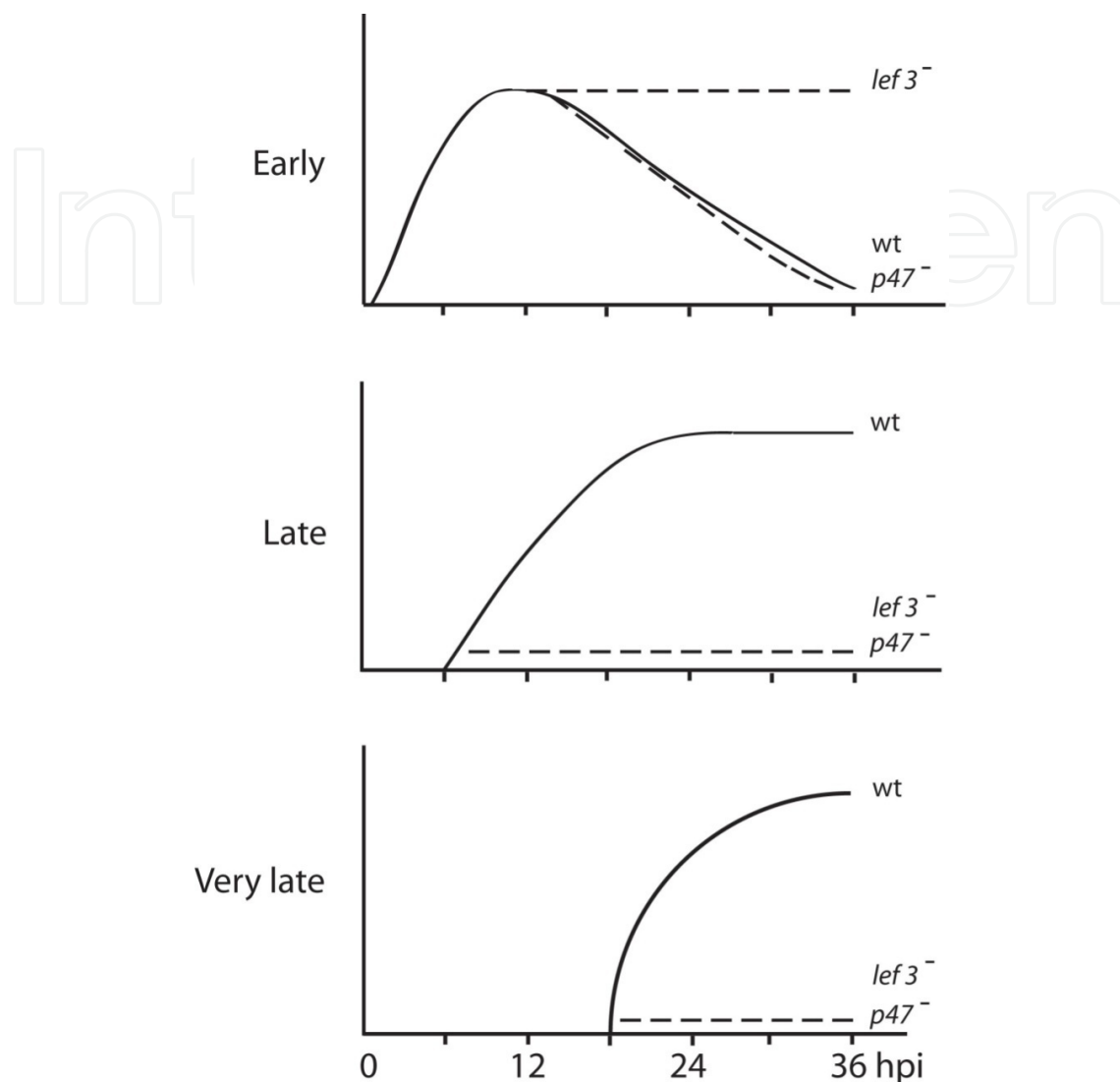


Figure 1. Kinetics of transcription of baculovirus genes. The time courses of steady-state level of transcripts (y axis) of the three AcMNPV temporal classes of genes, in Sf21 cells, are shown schematically. Solid lines represent mRNAs in wild-type (wt) infection; dotted lines represent idealized transcriptional profiles in infections in which a virus gene required for either DNA replication or late transcription was silenced by RNAi (denoted by "-"). Early genes are transcribed by the host RNA polymerase II before virus DNA replication, which initiates 6 to 9 hours post infection (hpi). Transcripts of early genes are detectable within 1 hpi, reach a maximum between 6 to 12 hpi and diminish thereafter (shutoff). Transcription of late and very late genes depends on viral DNA replication and is performed by a virus-encoded RNA polymerase. Transcription of late genes begins with the onset of DNA replication and continues mainly up to 24 hpi, while transcription of very late genes bursts around 18 hpi and continues through 72 hpi. The figure shows that late/very late gene transcription is abolished by silencing *p47*, a gene that encodes a subunit of the RNA polymerase, which is essential for the activity of the enzyme. A similar effect is shown as consequence of silencing *lef3*, a gene essential for virus DNA replication, since DNA replication is required for transcription of late/very late genes. However, the effects of silencing these genes on expression of early genes are different. When *p47* is silenced, early gene transcription declines as in wild-type infection; in contrast, silencing of *lef3* blocks the reduction of transcription, indicating that DNA replication, rather than late transcription, is a primary determinant of the shutoff of early genes (adapted from [65]).

5. Expression of early genes

At the time the virus reaches the nucleus, the template DNA for transcription as well as proteins of the virion that are carried over and may activate transcription are at their lowest levels. Therefore, the success of the infection depends on the ability of the virus to efficiently redirect the cellular system to express early genes encoded in its genome. Baculovirus DNA is infectious without any accompanying protein, as proved by the infection that follows after transfection of permissive cells with viral DNA. This indicates that baculovirus early promoters are responsive to the RNA pol II and accordingly, their structural organization resembles that of the host genes which are transcribed into mRNAs.

Promoters of baculovirus early genes consist in a core region and regulatory proximal sequences that may be recognized for specific binding of transcription factors from either the host or the virus. The core promoter includes two characteristic elements: a TATA-box-like sequence and a transcription initiator (INR), although one or both are absent in some early promoters [12]. The TATA box is the site for assembly of the preinitiation transcription complex by first binding of TATA binding protein (TBP). After the RNA pol II is recruited to the complex, transcription starts about 30 nucleotides downstream of the position of the TATA-box. The INR determines the starting site for transcription (nucleotide +1 of the primary transcript), and ensures proper initiation when there is no TATA-box present. CAGT is the most conserved INR sequence motif in baculovirus early promoters. Other activating sequences can be found either upstream of the core promoter or downstream, in the 5'UTR of the regulated gene.

Besides the sequences within the promoter region that modulate expression of a gene through the binding of regulatory proteins, there are sequences that enhance transcription from promoters even if they are located at a long distance. In baculoviruses there are non-coding regions known as homologous regions (*hr*) that play the role of enhancers [13] (see below).

Baculovirus early genes can be subdivided into two categories: immediate-early (*ie*) and delayed-early genes. Expression of *ie*-genes does not require viral factors, whereas the transcription of delayed-early genes was shown to need activation by *ie*-genes in transient expression assays. The major transactivator of AcMNPV early genes is the product of *ie1*, a gene that is present in all lepidopteran baculoviruses [14].

Other *ie*-genes known to regulate the expression of early genes are *ie2* and *pe38*. *ie2* is conserved only in group I alphabaculoviruses, one of the two phylogenetic lineages in which members of this genus can be separated. In transient expression experiments it was determined that *ie2* increases IE1-mediated transactivation of early promoters when *ie1* is present at low concentrations [15].

5.1. Immediate early transactivator IE1

AcMNPV IE1 is a 582 amino acids long protein exhibiting general characteristics of transcription factors (figure 2). It has a modular organization with domains associated to different functions: dimerization, nuclear import, DNA-binding, transactivation and replication [16-17].

Dimer formation is required for nuclear localization since the protein mutated in the dimerization domain cannot be imported into the nucleus [18]. IE1 transactivates early promoters including its own. Two mechanisms are postulated by which IE1 is capable of activating an early promoter: one independent and one dependent on DNA-binding. In the first one, IE1 activates transcription by interaction with cell factors recruited to the promoter regulatory regions. In the DNA-binding-dependent mechanism, the activation depends on binding of IE1 to *hr* sequences which function as enhancers. The *hrs* contain a variable number of imperfect palindromic repeats in tandem, separated by non-palindromic sequences. The palindromes are conserved within a genome but differ widely between genomes. In AcMNPV the imperfect palindrome consensus sequence has a length of 28 nucleotides with a central *EcoRI* site. According to the current knowledge, each subunit of the IE1 dimer interacts with a corresponding hemipalindrome during binding to an *hr*. Binding of IE1 to the enhancer increases the effective concentration of IE1 molecules able to interact with cellular factors in the promoter region. It was found that *hrs* also bind cellular factors in sites overlapping palindromic repeats and within interpalindromic regions [19-20]. *Hrs* are enriched in sequence motifs similar to cAMP and TPA response elements known to interact with transcription factors of the bZIP family. This is consistent with the ability of *hrs* to enhance transcription from baculovirus early promoters even in the absence of viral factors (see [21] for a review).

It has been reported that AcMNPV IE1 down-regulates the expression of certain genes. Promoters of these genes contain a sequence motif similar to one half of a typical *hr* palindrome which still functions as a target for IE1 binding; however, IE1 bound to this sequence is no longer able to promote activation, instead it functions as a repressor [22].

IE1 is the only known baculoviral gene that is expressed as part of a product of alternative splicing designated IE0. Compared to IE1, AcMNPV IE0 contains 54 additional aminoacids at its N-terminus. Both protein species are required for an efficient infection, although each one is dispensable given the other is present [23].

6. Expression of late and very late genes

Experiments using α -amanitin, an inhibitor of RNA pol II, showed that the synthesis of virus-specific mRNA becomes resistant to the drug with the progression of the infection, indicating that a novel RNA polymerase is induced in infected cells [24-25]. In AcMNPV-infected cells this viral encoded enzyme transcribes late genes mostly from 6 to 24 hpi and very late genes between 18 and 72 hpi. These times correlate with the production of BVs and ODVs, respectively. Expression of late genes depends on viral DNA replication. When replication is blocked with the DNA synthesis inhibitor aphidicolin, transcription of late genes is also inhibited [26]. In accordance to this dependency, whose nature is not known, genes involved in DNA replication are also required for transcription of late genes, and therefore, they are considered as a subset of the factors regulating late gene expression, collectively known as late expression factors (*lefs*). The remaining *lefs*, including those encoding the multi-subunit RNA polymerase, are considered to regulate transcription-specific events. AcMNPV *lefs* were discovered using

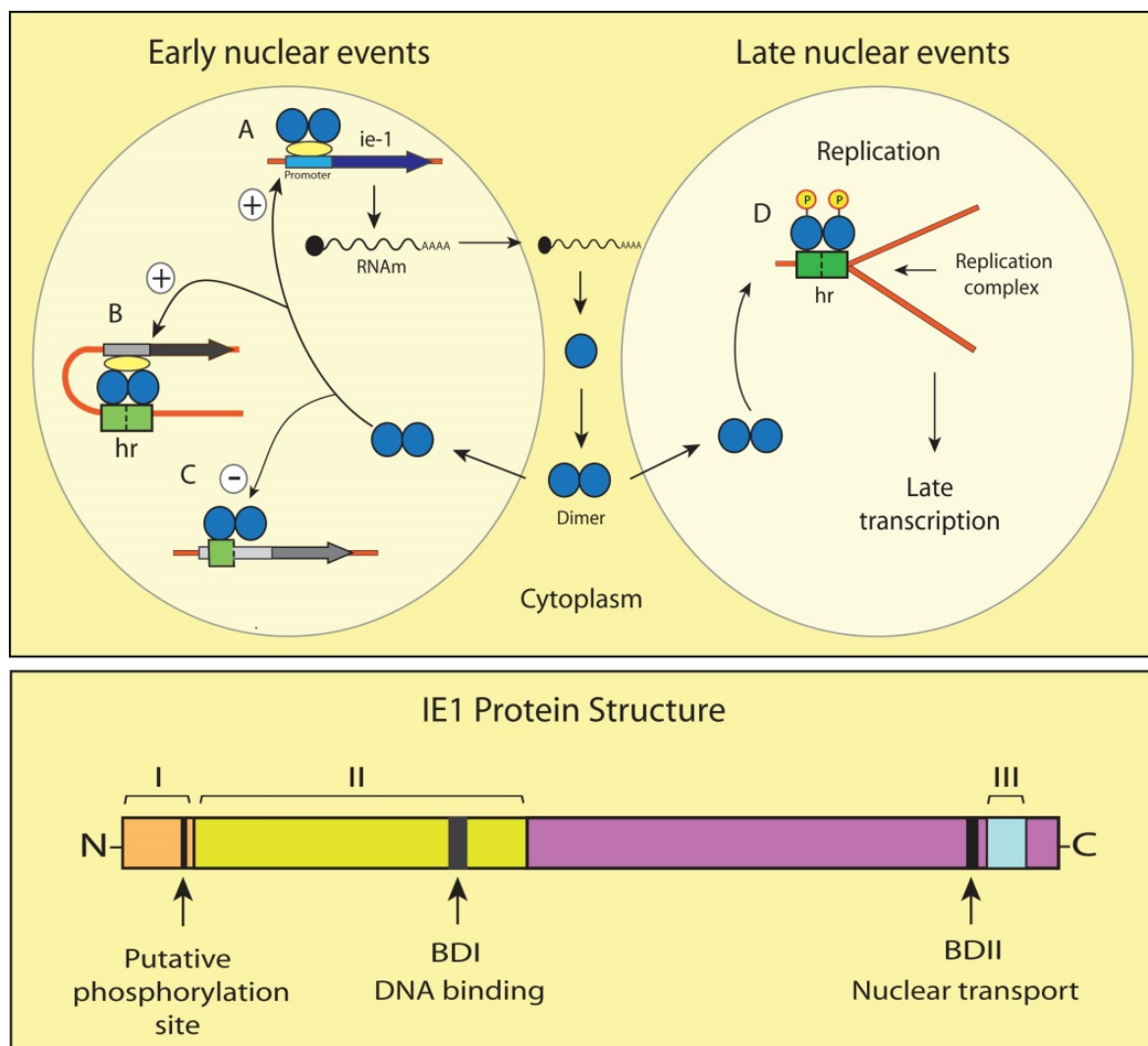


Figure 2. Functions of IE1 during the replicative cycle of AcMNPV. IE1 is targeted to the nucleus by a nuclear localization element (NLE) that becomes functional upon IE1 dimerization. The NLE is determined by a small basic domain (BDII) located at the C-terminus of the molecule adjacent to a helix-loop-helix dimerization domain. IE1 transactivates early promoters through interacting directly with transcription factors in the promoter (A) or via binding to *hr* enhancers (B). The *hr*-mediated transactivation mechanism depends on the interaction of the basic domain I (BDI) of each IE1 monomer in the dimer with a corresponding hemipalindrome of an *hr* repeat. IE1 may bind sequences within a promoter that resemble an *hr* palindrome half-site and down-regulate rather than activate transcription, as observed in transient expression assays (C). With the onset of viral DNA replication, early transcripts are down-regulated and there is a switch to the viral-encoded RNA polymerase which takes over transcription of late genes. At this stage, IE1 is required for the replication of virus DNA. *Hrs* may serve as origins of replication and it appears that binding of IE1 to *hrs* recruits requisite factors to assemble the replication complex (D). This IE1 function is associated with the N-terminal domain of the protein designated as the replication domain. This domain mediates phosphorylation of IE1 at the initiation of DNA replication. It has been proposed that this event is timely regulated and determines the functional switch of IE1 from transcription- to replication-associated activities. IE1 functional domains for replication, transactivation and dimerization, are indicated in lower panel with I, II and III, respectively (adapted from [37])

different approaches including temperature-sensitive mutations mapping and transient assays of plasmid DNA replication and late gene expression (for review see [8]). In transient expression assays, a plasmid containing a reporter gene under control of a late promoter was

cotransfected into cells with an overlapping clone library representing the AcMNPV genome [27]. Genomic DNA fragments containing *lefs* were identified as a consequence of the lack of reporter activity when they were omitted in cotransfections. Gradual shortening of those fragments led to the identification of each *lef*. Nineteen AcMNPV *lefs* were identified as required for activation of the late promoter in this system: *lef1* to *lef12*, *ie1*, *ie2*, *dnapol*, *p143*, *p35*, *p47* and *39k* [28] (Table 1). In addition to the *lefs*, a gene designated *vlf1* was found necessary to support expression from a very late promoter. Some other genes influence DNA replication or late gene expression either directly or indirectly, and may be considered as *lefs* also [8]. Differences in reports on the relative contribution of specific genes appear to be consequence of different experimental approaches utilized in studies.

ORF name	ORF number	Amino acid residues	Homologs in baculovirus lineages [§]	Functional class	LEF function
<i>lef1</i>	14	266	core	Replication	DNA primase
<i>lef2</i>	6	210	core	Replication	primase accessory protein
<i>dnapol</i>	65	984	core	Replication	DNA polymerase
<i>p143</i>	95	1221	core	Replication	DNA helicase
<i>lef11</i>	37	112	α-I, α-II, β, γ	Replication	
<i>lef3</i>	67	385	α-I, α-II, β	Replication	ssDNA binding protein
<i>ie1</i>	147	582	α-I, α-II, β	Replication	transactivator of early genes, <i>hr</i> -binding protein
<i>lef7</i>	125	226	α-I, α-II*, β*	Replication	possible ssDNA binding protein
<i>ie2</i>	151	408	α-I	Replication	transactivator of early genes, cell-cycle control
<i>p35</i>	135	299	α-I*, α-II*, β*	Replication	apoptosis inhibitor
<i>lef4</i>	90	464	core	Transcription	RNA polymerase subunit, capping enzyme,
<i>lef8</i>	50	876	core	Transcription	RNA polymerase subunit
<i>lef9</i>	62	490	core	Transcription	RNA polymerase subunit
<i>p47</i>	40	401	core	Transcription	RNA polymerase subunit
<i>lef5</i>	99	265	core	Transcription	transcription initiation factor
<i>lef6</i>	28	173	α-I, α-II, β	Transcription	
<i>39k/pp31</i>	36	275	α-I, α-II*, β	Transcription	DNA binding protein
<i>lef10</i>	53A	78	α-I, α-II*, β*	Transcription	
<i>lef12</i>	41	181	α-I, α-II*	Transcription	

[§] Core genes have homologs in all sequenced baculoviruses. α-I, α-II: Group I and Group II of alphabaculoviruses, respectively. β: betabaculoviruses, γ: gammabaculoviruses.

*There is at least one member in the indicated group having an homolog of the corresponding gene.

Table 1. AcMNPV *lefs*

6.1. Replication *lefs*

Among *lefs* involved in viral DNA replication [29-30], four baculovirus core genes are essential for this process: *lef1*, *lef2*, *dnapol* and *p143*; they were required in transient assays for plasmid replication as well as for late gene expression. *lef1* is a DNA primase that interacts with *lef2*, a primase accessory protein [31]. *dnapol* encodes a DNA polymerase with 3' to 5' exonuclease activity. The sequence of *dnapol* is the most conserved among baculovirus replication *lefs*; the phylogeny of the family Baculoviridae inferred from its sequence is congruent with that resulting from the analysis of all core genes altogether, suggesting that *dnapol* is an ancestral gene [32]. P143 is a helicase that requires ATP to unwind DNA. Two other AcMNPV replication *lefs*, *ie1* and *lef3*, which are present in all lepidopteran baculoviruses, are also essential as determined in transient assays. Nevertheless, their absence in the genome of γ - and δ -baculoviruses suggests that there may be functional homologs of these genes involved in replication/gene expression in species belonging to these genera. LEF3 is a single stranded DNA-binding protein that promotes unwinding of DNA duplex and annealing of complementary strands [33]. LEF3 interacts with p143 and shuttles this molecule to the nucleus [34].

The actual function of IE1 in DNA replication is poorly understood, nonetheless it appears to depend on the *hr*-binding capacity of IE1. *Hr* regions function not only as enhancers of early genes but also serve as origins of DNA replication in plasmid replication assays [35]. It was shown that in the presence of an *hr* element, transiently expressed IE1 adopts a focal distribution within the nucleus. When LEF3 and P143 are simultaneously expressed they localize to the *hr*-induced IE1 foci [36]. This suggests that IE1 functions by recruiting viral replication factors to the *hr* origin. The switch to the replication activity of IE1 seems to be timely regulated by phosphorylation [37].

Other genes have a stimulatory effect in transient replication/late expression assays. These are *ie2*, *lef7* and *p35*. IE2 is a transactivator involved in cell cycle arrest [38] and LEF7 has sequence similarity to single stranded-DNA binding proteins. Both are present in all genomes of group I α -baculoviruses and LEF7 is also present in some other α - and β -baculoviruses. P35 blocks the apoptotic response of cells triggered by DNA replication through inhibition of effector caspases [39]. The contribution of P35 in transient assays reflects its protective effect against apoptosis, and therefore represents an indirect requirement for replication and late gene expression. Actually, it may be replaced in the assays by a member of the baculovirus *iap* (inhibitor of apoptosis) gene family with similar results. *ie2*, *lef7* and *p35* are dispensable for the infection of TN368 cells by AcMNPV [40]; hence, they are considered as host range factors able to extend the infectivity of AcMNPV towards Sf21 cells.

AcMNPV *lef11*, which was necessary for late gene expression in transient assays, was not required in transient plasmid replication assays. However, an AcMNPV *lef11*-null bacmid was unable to replicate its DNA [41]. Therefore, *lef11* is essential for DNA replication in the context of the virus infection and may be considered as a replication *lef*.

6.2. Transcription lefs

The viral RNA polymerase is a complex of the products of four baculovirus core genes: *lef4*, *lef8*, *lef9* and *p47*. LEF8 and LEF9 have motifs present in the two large subunits of RNA polymerases from prokaryotes and eukaryotes, and are supposed to participate of the catalytic domain. Their sequences are the most highly conserved among LEFs. The role of LEF4 as a capping enzyme is discussed below, while the specific function of P47 remains to be elucidated. The polymerase complex was chromatographically isolated as a fraction that was active in *in vitro* transcription assays using template DNA containing signals specific of baculovirus late gene promoters [42]. In the complex, the four subunits are present in an equimolar ratio.

lef5 is a core gene that encodes a protein with sequence similarity to the eukaryotic transcription elongation factor TFIIS, however, *in vitro* assays evidenced the ability of LEF5 to increase the transcriptional activity of the viral RNA polymerase at the initiation step rather than to have any effect in the elongation process [43]. The remaining *lefs* have a distribution among species restricted to lepidopteran baculoviruses. *39k* (also known as *pp31*) and *lef10* are considered essential for late gene expression although their specific role in transcription is not known. 39K binds single- and double-stranded DNA and localizes to the virogenic stroma during infection. LEF10 is a small polypeptide without homology to known proteins. *lef6* and *lef12* are considered auxiliary lefs because although they were shown necessary in transient assays (*lef12* is dispensable in TN368 cells), knockout mutants for these genes sustained late gene expression with minor deviations from wild type virus [44-45].

6.3. Late and very late mRNAs synthesis and regulation

Promoters of late genes contain a TAAG sequence motif from which transcription is initiated. There are less TAAG motifs in baculovirus genomes than expected by random occurrence, suggesting that the activity of this sequence as a late promoter selects negatively its random distribution. The integrity of this motif is strictly necessary for transcription, while adjacent sequences up to eighteen nucleotides may affect the level of expression [46]. There may be more than one functional TAAG over a variable distance upstream the translational start codon of the regulated gene [47].

Late transcripts usually span more than one ORF; likewise, one specific ORF may be represented in transcripts with different 5' or 3' ends. The significance of these polycistronic messages is not known and it is generally assumed that only the leading gene in the message is translated into protein. Late genes are encoded in both DNA strands, distributed over the genome, therefore there may be opposite late transcripts with complementary stretches. It is not known if this may play any regulatory role considering that baculovirus genes are susceptible to silencing by double-stranded RNA [48]. Late transcripts are capped and polyadenylated at their 5' and 3' ends, respectively. At least two enzymatic activities required for capping reside in the LEF4 subunit of the RNA polymerase. This protein functions as RNA triphosphatase and guanylyltransferase but lacks activity of N7-methyltransferase, which is required for methylation of the cap structure in position N7 of guanine [49-50]. A gene responsible for this activity has not been identified in baculovirus. The structure of cap 1 mRNAs includes methylation of the 2'hydroxyl group of the ribose of the first transcribed

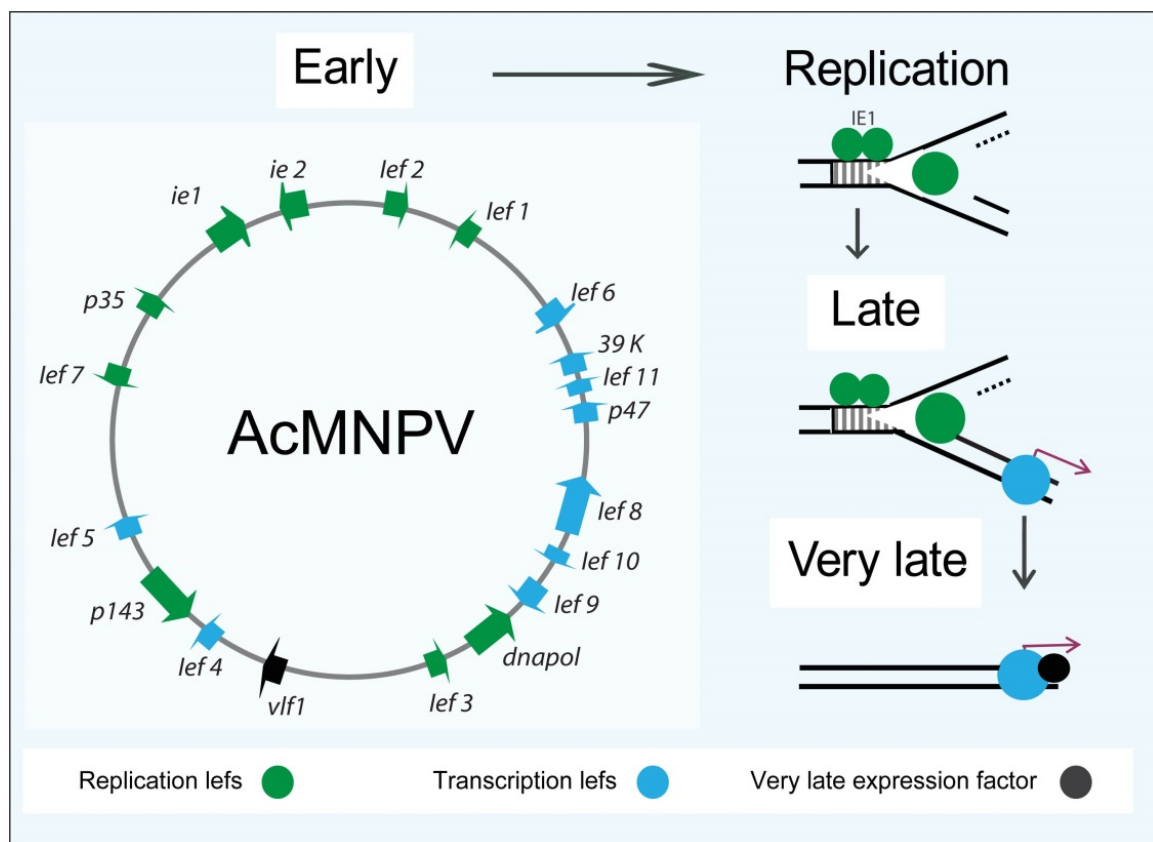


Figure 3. Cascade of baculovirus gene transcription events. Diagram of the AcMNPV genome indicating the localization of the genes encoding key proteins involved in the regulatory network of transcription and DNA replication.

nucleotide by an RNA cap 2'O-methyltransferase (MTase-I). Several alphabaculoviruses have a MTase-I gene. The gene of AcMNPV has been found to stimulate late gene expression in transient assays [51]. Late transcripts are terminated by the polymerase at U-rich sequences present in their 3'UTR, and subsequently the enzyme adds adenosine residues independently of template [52].

The most expressed very late genes in AcMNPV are those encoding polyhedrin and P10. Their transcription depends on a TAAG initiation promoter but their high level of expression depends on the presence of an AT-rich sequence known as the "burst" sequence, located between the TAAG and the translational start codon [53-54]. The burst sequence binds very late expression factor-1 (VLF1; [55]) originally identified in a temperature sensitive AcMNPV mutant defective in occluded virus production [56]. VLF1 is a baculovirus core gene that is essential for the packaging of DNA into normal nucleocapsids.

7. Cellular responses to infection and changes in host gene expression

Early in infection, baculoviruses produce cell cycle arrest at G2/M or S phase, prior to viral DNA replication [57]. The AcMNPV early transcription coactivator IE2 is considered to be

involved in regulation of cell-cycle [38]. The progression of infection is accompanied by profound changes in the expression of cellular genes. The host protein synthesis is shutoff starting at around 12-18 hpi [58]. This was found to be mostly the result of a reduction in the levels of transcripts rather than in translation of mRNAs [59], though the actual mechanism of the decrease in the steady-state level of host messages is not known.

Despite host genes are eventually down-regulated at late times, Nobiron and co-workers [60] found that the transcript of a cognate heat shock protein (hsc) 70 gene was transiently up-regulated early in AcMNPV-infected Sf9 cells. In a comprehensive study of gene expression profile of Sf21 cells using microarrays designed from an EST database of *S. frugiperda*, Salem and co-workers [61] confirmed the general shutoff of host transcription over time of AcMNPV infection, but interestingly, they found that about 25% of host genes were slightly up-regulated at 6 hpi. The expression of heat shock proteins (HSPs) of the 70-kDa family in infected cells was followed by western analysis [62]. The results of this study showed changes in the cellular pattern of HSP/HSC70s. Moreover, the infection potentiated the response to heat shock, boosting the HSP/HSC70s content of cells several-fold in comparison with uninfected cells.

The actual level of cellular proteins during infection may vary with a different kinetics of that of the steady-state level of their mRNAs. For example, in a study by Rasmussen and Rohrmann [63], the level of TBP in AcMNPV-infected Sf9 cells, revealed constant until 72 hpi. In other study, TBP was actually found to increase between 16 and 72 hpi in Sf21 and TN368 cells, and to co-localize with viral DNA replication centers within the nucleus [64]. Therefore, TBP appears not be targeted for degradation as it is in other viral systems. However, the functional significance of its increment is unclear, given that it coincides with decreasing levels of transcripts synthesized by RNA pol II.

Currently, due to the relevance of AcMNPV as vector for the expression of proteins in cultured insect cells, it is of special interest to understand the global shutoff of host protein synthesis. In this system, the expression of foreign proteins is driven by the promoter of polyhedrin gene, which is most active at very late times of infection. By this time many processes and pathways appear highly compromised, and the expression of certain classes of proteins may be severely affected, especially those involved in trafficking through the ER and Golgi.

Baculoviruses induce apoptosis of infected cells [39]. Programmed cell death functions as an antiviral defense response to prevent production of virus progeny and spreading of the infection. To counteract the apoptotic response, baculoviruses encode antiapoptotic genes. P35 is a potent antiapoptotic protein of AcMNPV that inhibits the activity of effector caspases. The results of experiments using an AcMNPV *p35* mutant that causes apoptosis upon infection in Sf21 cells showed that apoptosis is triggered by replication of the viral DNA [65]. Apoptosis induced by this mutant was inhibited when each one of the AcMNPV genes required for replication was independently silenced by RNAi. Silencing of these genes also inhibited shutoff of host proteins synthesis, suggesting that both processes are linked. These cellular responses resemble that of vertebrates which arises as consequence of cell cycle arrest or DNA damage. In a recent report Huang and co-workers [66] presented evidence indicating that infection of Sf9 cells with AcMNPV induces a DNA damage response which is required for efficient replication of the virus.

8. Baculoviral microRNAs

MicroRNAs (miRNAs) are small RNA molecules of ~20-22 nt that regulate gene expression posttranscriptionally in a sequence dependent way. miRNAs have been widely described in animals and plants and regulate expression of protein coding genes involved in numerous processes. Genes coding for miRNAs are transcribed by the RNA pol II. The primary transcript (pri-miRNA) containing a hairpin loop is processed by the RNase III-like enzyme Drosha releasing the precursor miRNA (pre-miRNA). The pre-miRNA is a ~80 nt molecule that contains an imperfect hairpin loop and is exported to the cytoplasm by Ran-GTP dependent Exportin 5. Once in the cytosol, the pre-miRNA loop is cleaved by another RNase III enzyme, Dicer, leaving the RNA duplex consisting of the mature miRNA and its complement (miRNA*). One of these strands (the mature miRNA) is then incorporated in the RNA-Induced Silencing Complex (RISC), which is then ready to target the specific mRNA and either represses its translation or degrades it [67].

Viruses were also found to encode miRNAs. Strikingly, nearly all the virus encoded miRNAs were reported from DNA viruses, especially those that have a nuclear cycle, with access to the microRNA processing proteins. The majority of the viral miRNAs described belong to herpesviruses. Interestingly, studies of virus-host interactions revealed a complex miRNA regulation with both viral and host microRNAs regulating both viral and host mRNA targets [68-69]. Regarding insect viruses miRNAs, little is known yet. Two viruses, belonging to *Ascoviridae* and *Baculoviridae*, were reported to code for miRNAs. The first report of a miRNA encoded by an insect virus was from the *Heliothis virescens* ascovirus (HzAV). This virus codes for a miRNA that targets viral DNA polymerase and regulates viral replication [70]. More recently, Singh and colleges [71] presented a study in which they found and validated four miRNAs encoded by *Bombyx mori* nucleopolyhedrovirus (BmNPV): *bmnpv-miR-1*, *-2*, *-3* and *-4*. This was achieved by sequencing small RNAs obtained from infected tissues of *B. mori* larvae followed by *in silico* analysis and validation using northern blot hybridization, stem-loop RT-PCR and poly(A)-tailed RT-PCR. Interestingly, closely related baculoviruses were found to contain these miRNA in their genomes in conserved positions. All four BmNPV miRNAs are present with 100% identity in AcMNPV, BomaNPV and PlxyMNPV whereas three miRNAs were conserved in RoMNPV and only one in MaviNPV. In contrast of what occurs in animals and plants (miRNAs coded in intergenic regions or introns), these microRNAs were found in genomic locations completely overlapping viral ORFs, either in the coding or the complementary strand. *In silico* predictions revealed putative targets, either viral or from the host. Viral predicted targets include *dna binding protein*, *chitinase*, *bro-I*, *bro-III*, *lef8*, *fusolin*, *DNA polymerase*, *p25* and *ORF 3* of BmNPV. Cellular predicted target genes encode proteins related to antiviral defense mechanisms, such as prophenoloxidase and hemolin, or proteins that play an important role in small RNA-mediated gene regulation like GTP binding nuclear protein Ran, DEAD box polypeptides and eukaryotic translation initiation factors [72].

A further study on *bmnpv-miR-1* revealed the sequence dependent interaction of this miRNA with cellular Ran mRNA. The GTP-binding nuclear protein Ran is an essential component of the Exportin-5-mediated nucleocytoplasmic transport machinery involved in the transport of

small RNAs from the nucleus to cytoplasm. Downregulation of Ran by the expression of *bmnprv-miR-1* in viral infection triggers the reduction of the host small RNA population and increasing of the viral load in infected *B. mori* larvae. In this way BmNPV counteracts the small RNA mediated defense of its host for its effective proliferation [63].

9. Persistent infections

It is known that some viruses are capable of persisting in their hosts without causing disease. This can be accomplished by producing either a latent or a persistent infection. The main difference between both is that during latent infection the virus is not replicating and keeping a minimal gene expression while in persistent infections all the genes are expressing, at low levels, without causing any symptom. Herpesviruses can establish latent infections in specific cell types [73]. This state is characterized by a unique transcriptional program that involves the expression of latency-associated transcripts (LATs) as the only viral products synthesized in large quantities. The virus is maintained as an independent quiescent genetic material within the host cell nucleus. An alternative mechanism is observed in measles virus by which the virus remains at low levels with the production of viral proteins. This is usually referred to as persistent infection [74].

The White Spot Syndrome Virus (WSSV) is a non-occluded virus pathogenic to shrimp, phylogenetically related to baculoviruses. It was found at very low levels in asymptomatic shrimps. The virus may reside within cells in a quiescent state as in a latent infection or causing a persistent infection [75]. Similarly, a nudivirus was found infecting persistently the cell line IMC-Hz1, derived from the corn earworm *Heliothis zea*.

Baculoviruses are highly lytic, causing a lethal disease in infected larvae. Epizootics caused by these viruses can reduce dramatically their host population [76]. Persistence of baculoviruses in the environment is mainly thought to be due to the OBs that protect virions from UV light and allow horizontal transmission. But there seems to exist another way for baculoviruses to persist in the environment at low host densities. Baculoviruses can cause sublethal infections, and so be vertically transmitted from adult to offspring [77-79] or may as well become persistent or latent [80]. A laboratory colony of *Mamestra brassicae* was found to harbour an occult infection by the baculovirus MbMNPV with expression of viral genes at a low level [81]. Later, Burden *et al.* demonstrated the persistence of this virus in naturally occurring field populations of *M. brassicae*. RT-PCR analysis showed the presence of *polyhedrin* transcripts in asymptomatic larvae, indicating a covert infection [80-81]. Similar results were obtained using *ie1* as a target [81]. Moreover, these studies revealed that covert infections could be induced to produce overt infections when infecting these larvae with another baculovirus. This means that the persistently infecting virus retains its ability to produce a lethal disease in the larva.

There is accumulating evidence of persistent baculoviral infections. Kemp *et al* [83] detected baculoviral presence (CfMNPV, CfDEFMNPV and a GV) in laboratory and field populations of *Choristoneura fumiferana*. Also, there were baculoviruses (SeMNPV and MbNPV) identified in *Spodoptera exigua* populations that could be reactivated to full lethal forms [84]. A study in

field populations of *Spodoptera exempta* showed that virtually all the insects collected in the field were positive for *S. exempta* nucleopolyhedrovirus (SpexNPV) DNA and 60% of these insects had transcriptionally active virus, suggesting that SpexNPV is transmitted vertically at extremely high levels in field populations of *S. exempta* and can maintain a persistent infection without obvious symptoms [85].

On the whole, baculoviruses seem to use different strategies to persist in nature: on one hand OBs permit their subsistence outside the host for horizontal transmission while, on the other hand, they can persist as covert infections in the host, allowing vertical transmission too. Moreover, these covert infections can be triggered to overt infections producing the typical lethal disease in the host. Nevertheless, the mechanisms of reactivation of these sublethal infections remain to be elucidated.

10. Concluding remarks

Among nuclear DNA viruses, baculoviruses have developed a unique strategy to synthesize late mRNAs which consists in having their own DNA-directed RNA polymerase. This enzyme recognizes viral late promoters that are different to promoters responsive to the cellular RNA polymerase. By this means, the infected cell produces high levels of viral proteins at times of the infectious cycle at which the cellular protein synthesis is mostly shutdown. A late viral progeny with a distinct phenotype is embedded in proteinaceous occlusion bodies (OBs) that assemble after overexpression of the major OB protein. In order to exploit their high protein synthesis capacity, baculoviruses have been developed as vectors for expression of heterologous proteins in insect cells. This system is continuously evolving to new biotechnological applications. However, there is still a lack of knowledge about the molecular mechanisms governing the complex baculovirus infectious cycle. A better understanding of these mechanisms would also benefit the development of baculovirus as biopesticides. To this regard, the array of viral factors involved in regulation of gene expression is an important component of the specific virus-host interactions that determinate the susceptibility to the virus of different cell types within a host and of different hosts within a range of insect species.

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