Chapter 2

Introduction to Baculovirus Molecular Biology

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Abstract

The development of baculovirus expression vector systems has accompanied a rapid expansion of our knowledge about the genes, their function and regulation in insect cells. Classification of these viruses has also been refined as we learn more about differences in gene content between isolates, how this affects virus structure and their replication in insect larvae. Baculovirus gene expression occurs in an ordered cascade, regulated by early, late and very late gene promoters. There is now a detailed knowledge of these promoter elements and how they interact first with host cell-encoded RNA polymerases and later with virus-encoded enzymes. The composition of this virus RNA polymerase is known. The virus replication process culminates in the very high level expression of both polyhedrin and p10 gene products in the latter stages of infection. It has also been realized that the insect host cell has innate defenses against baculoviruses in the form of an apoptotic response to virus invasion. Baculoviruses counter this by encoding apoptotic-suppressors, which also appear to have a role in determining the host range of the virus. Also of importance to our understanding of baculovirus expression systems is how the virus can accumulate mutations within genes that affect recombinant protein yield in cell culture. The summary in this chapter is not exhaustive, but should provide a good preparation to those wishing to use this highly successful gene expression system.

Key words Baculovirus, Gene expression, Promoters, Insect cells, Virus structure, Virus replication, Apoptosis

1 Introduction

The last 20 years have seen baculoviruses maintain a reputation for producing high yields of recombinant proteins in insect cells. Despite the perceived difficulties of working with a virus in eukaryotic cells, the fact that posttranslational modifications such as glycosylation, fatty acid acylation, disulphide bond formation, and phosphorylation are carried out very similar to the same processes in mammalian cells has convinced many users of the value of baculoviruses as expression vectors. Continuous development of the system by many groups has seen the early problems of making recombinant viruses a distant memory. Using baculoviruses as expression vectors is no longer the preserve of specialist virologists.

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The many commercial kits now available have brought the system within the capabilities of any competent molecular biologist. Most of these kits are based on the prototype member of the Baculoviridae, namely *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and utilize the highly expressed polyhedrin gene promoter. The simplicity of culturing insect cells such as *Spodoptera frugiperda* or *Trichoplusia ni* makes scale up of recombinant protein production feasible for most laboratories. The problems experienced with spinner or suspension cultures where shear forces limited viability have largely been solved with the introduction of serum-free media allied with antifoam and protective (e.g., Pluronic® F-68) agents. The exploitation of baculoviruses as biosafe insecticides has also benefited from the work on expression vectors and fundamental studies on virus gene function. While most baculovirus expression vectors lack the original polyhedrin gene required for making occluded viruses, recombinant virus insecticides can preserve this process by utilizing nonessential regions of the virus genome for the insertion of foreign genes encoding insecticidal proteins.

2 Classification

The Baculoviridae are a family of DNA viruses with circular double stranded genomes that only infect arthropods. They are characterized by their ability to occlude virions in a crystalline protein matrix to form either polyhedra or granules. Viruses forming polyhedra are known as nucleopolyhedroviruses (NPVs) and those forming granules as granuloviruses. This subdivision is based on a number of criteria, including occlusion body morphology and the mechanism by which nucleocapsids are enveloped in infected cells [1]. Granuloviruses produce small occlusion bodies (OBs) ($0.16-0.30\ \mu m \times 0.30-0.50\ \mu m$) normally containing one or occasionally two virions encapsulated in a protein called granulin. Nucleopolyhedroviruses produce larger occlusion bodies (0.5–1.5 μm in diameter) composed of polyhedrin protein, which contain many virions. The family is divided into four genera that only infect arthropods. The Alphabaculoviruses and Betabaculoviruses comprise nucleopolyhedroviruses (NPVs) and granuloviruses (GVs) that are isolated from Lepidoptera (butterflies and moths). Figure 1 illustrates an example of each of these genera. Deltabaculoviruses and Gammabaculoviruses comprise NPVs and infect dipteran (flies) and hymenopteran (sawflies) species, respectively. The NPVs pathogenic for members of the order Lepidoptera have been further subdivided into groups I and II based on molecular phylogenies [1, 2]. An occluded virus also infects the pink shrimp *Penaeus monodon* [3–5]. Partial sequence analysis showed that it encodes proteins similar to those produced by baculoviruses.

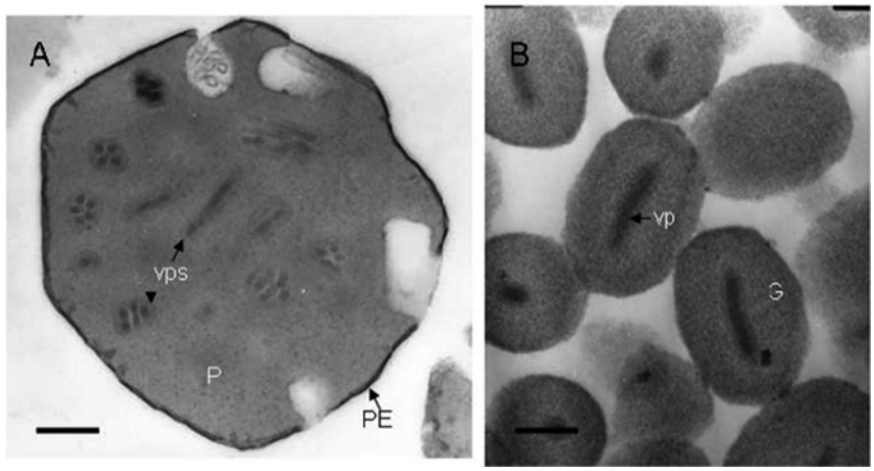

Fig. 1 Electronmicrographs of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) polyhedron (**a**) and *Plodia interpunctella* granulovirus (PiGV) granules (**b**). Note the multiple virus particles (vps) in longitudinal or cross section in AcMNPV, whereas in PiGV each granule contains a single virus particle (vp). Virus particles are occluded within polyhedrin protein (P) or granulin protein (G). Polyhedra are bounded by an envelope (PE), which contains protein and carbohydrate. Scale bar is 200 nM

3 Baculovirus Structure

The double stranded, supercoiled, circular DNA genome of AcMNPV is highly condensed within a nucleocapsid. This condensed packaging is facilitated by the core protein p6.9, a 54 amino acid protein rich in arginine [6]. A major protein of the capsid, vp39, has also been identified. This 39 kDa protein has been found to be distributed randomly over the surface of the nucleocapsid [7].

There are two types of baculovirus progeny, budded virus (BV) and occlusion-derived virus (ODV), which are genetically identical [8]. However, there are differences in morphology, timing and cellular site of maturation, structural proteins, source of viral envelopes, antigenicity, and infectivity [9–13].

BV particles possess spike-like structures known as peplomers, composed of the glycoprotein GP64 for group I NPVs, at one end of the virion [14]. The GP64 protein is incorporated throughout the virus envelope, albeit at lower concentrations than at the peplomers [14]. During infection, GP64 localizes to discrete areas of the plasma membrane at which points budding of virions takes place [15]. Thus, as BV particles bud from the plasma membrane they acquire a plasma membrane-derived envelope containing the GP64 glycoprotein.

Granuloviruses and group II NPVs lack GP64 but instead encode a homolog of another envelope protein, LD130 [16]. While the LD130-encoding baculoviruses do not contain *gp64*

homologs, those encoding GP64 also contain a version of LD130. The OpMNPV LD130 homolog was found to be a glycosylated protein associated with BV particles and the plasma membrane of cells at similar locations as the GP64 protein [17].

One of the major differences between BV and ODV is that the latter do not contain GP64 or LD130. Instead a virus-encoded envelope protein P74 is associated with the outside of the virus [18, 19]. The presence of P74 on the outside of the virion envelope suggests that it may play a role in ODV attachment to midgut cells. Another structural protein of the ODV envelope of *Spodoptera littoralis* (Spli) NPV has been identified. The gene encoding this protein was termed *per os* infectivity factor (*pif*) and is homologous to ORF 119 of AcMNPV [20]. A third gene product inferred to be a structural protein is encoded by *pif-2*, which was identified in *Spodoptera exigua* NPV and is present in AcMNPV as ORF22 [21]. Recent studies have also identified *pif*-4 [22] and *pif*-5 [23, 24] as additional envelope proteins. The PIFs form a complex on the surface of ODV [25].

Other ODV-specific envelope proteins include ODV-E25 [26], ODV-E66 [27], ODV-E56 [28], ODV-E18, and ODV-E35 [29]. These proteins, along with P74, have been found to associate with intranuclear microvesicle structures, which appear in the nucleus during infection [29, 30]. This association has led to the suggestion that these microvesicles play a role in ODV envelopment [27, 28]. Invagination of the inner nuclear membrane observed during baculovirus infection has raised the possibility that it may act as the source of the microvesicles [31–33].

Another ODV-specific protein, GP41, has been identified as an O-linked glycoprotein, predicted to localize to the region between the virus envelope and the nucleocapsid, an area referred to as the tegument [34]. Although GP41 is not present in the budded form of the virus, it has been shown to be required for release of nucleocapsids from the nucleus during BV production [35]. An additional protein, ODV-EC27, has been identified as a structural protein of the ODV envelope and capsid, and may be present in a modified form in BV [29]. This protein may be a cyclin homolog, involved in host cell cycle arrest during baculovirus infection [36]. An envelope protein of both BV and ODV has also been identified (BV/ODV-E26) and found to be associated with intranuclear microvesicles [37]. A seminal study of protein composition of ODV was conducted by using a combination of techniques [38]. Screening expression libraries with antibodies generated to ODV or BV and mass spectroscopic (MS) analysis of ODV protein extracts identified up to 44 potential components unique to ODV. This topic was revisited recently when the protein content of BV was reexamined [39].

The major component of the NPV occlusion body is the 29 kDa polyhedrin protein. While the protein is thought to be small

enough to diffuse through nuclear pores, a nuclear localization signal (KRKK) has been identified at amino acids 32–35 [40]. Another region of the protein (amino acids 19–110) is required for assembly into large occlusion-like particles [40]. The polyhedrin protein is resistant to solubilization except under strongly alkaline conditions and functions to shield virions from physical and biochemical decay while outside the insect host, as well as protecting against proteolytic decay during late stages of infection [41].

Surrounding the polyhedral matrix of a mature occlusion body is the polyhedron envelope (PE), also known as the polyhedron membrane or calyx. The PE was originally reported to be composed of carbohydrate [42]; however, a 34 kDa phosphorylated protein (PP34) has since been found to be covalently bound to the PE of AcMNPV via a thiol linkage [43]. The polyhedron envelope is thought to increase the stability of the occlusion body in the environment and has been found to protect occlusion bodies from physical stress [43, 44]. A recombinant virus unable to produce PP34 was found to be deficient in polyhedron envelope formation and electron dense spacers, which are thought to be precursors of the envelope structure [44, 45].

Both electron dense spacers and the polyhedron envelope have been found in association with fibrillar structures composed of the P10 protein [44, 46]. This protein, like polyhedrin, is produced in large quantities during the occlusion phase. The association of the polyhedron envelope with the P10-containing fibrillar structures suggests a role for the protein in assembly and proper association of the polyhedron envelope around the occlusion body matrix [46]. Comparative analysis of P10 protein sequences revealed that they appear to have an amphipathic alpha-helical terminus that condenses as coiled-coil multimers [47]. The condensation of P10 monomers to coiled-coil multimers may be a step leading to fibrous body formation in virus-infected cells.

4 Baculovirus Replication In Vivo

Within a cell, baculoviruses have a biphasic cycle of replication producing two structurally distinct viral phenotypes responsible for specialized functions within the insect host. Occlusion-derived virus (ODV) initiates the primary infection in the gut epithelial cells, while budded virus (BV) is responsible for secondary rounds of infection in the insect host. Infection begins with ingestion of occlusion bodies by a suitable insect host larval stage, followed by dissolution of the occlusion body matrix in the alkaline midgut. Occlusion-derived virus is released from dissolved occlusion bodies, passes through the peritrophic membrane of the midgut and infects the columnar epithelial cells that border the gut lumen. It has been suggested that entry occurs by direct membrane fusion,

involving interaction between virion surface proteins and midgut cell surface proteins [48]. Removal of P74, PIF1 or PIF2 from virus particles by deletion of the appropriate gene has been demonstrated to prevent infection of insect larvae via the oral route [18–21]. Deletion of *pifs*-3-5 has a similar effect [22–24, 49]. Midgut regenerative cells have also been found to be sites of primary infection, albeit at a lower frequency than columnar cells [50]. After replication in the epithelial cells, virus buds in a polar manner, exiting the basal membrane into the hemolymph, thereby allowing the infection to spread throughout the insect host. Early synthesis of the BV envelope fusion protein GP64 seems to allow a "pass through" strategy for the virus, whereby uncoated virus nucleocapsids can rapidly exit the newly infected midgut cells to accelerate the onset of systemic infections [51]. The insect tracheal system has been identified as the main route used by the virus to spread from one tissue to another [52]. Efficient BV formation in AcMNPV requires Ac92 and Ac79 [53, 54].

In addition to enlargement of the nucleus upon baculovirus infection, cells become rounded due to rearrangement of the cytoskeleton. A distinct structure termed the virogenic stroma develops in the nucleus. This is a chromatin-like network of electron-dense filaments. It is the predominant structure in the nucleus from 8 to 48 h postinfection (h pi) [55], and is thought to be the site of viral DNA replication and late gene transcription, as well as nucleocapsid formation [56]. Once assembled, nucleocapsids are released from the nucleus, gaining a nuclear membrane-derived envelope that is subsequently lost during transit through the cytoplasm. In the case of group I NPVs, the envelope glycoprotein GP64 localizes to discrete areas of the plasma membrane during infection and it is at these sites that budding of nucleocapsids takes place [15]. The BV envelope is therefore derived from the plasma membrane and contains the viral glycoprotein GP64. Group II NPVs, and GVs, encode homologs of the envelope fusion (F) protein LD130 [16], which associate with BV particles, and the plasma membrane of infected cells, at similar positions to GP64 in group I NPV-infected cells [17]. These F proteins are functionally analogous to GP64 since they can restore infectivity to mutant viruses lacking *gp64* [57]. GP64 is essential for cell-to-cell BV transmission, since AcMNPV particles containing the protein, but not those lacking the protein, are able to disseminate virus to other cells [58].

Later in infection (~24 h pi), BV production is reduced and nucleocapsids are transported to sites of intranuclear envelopment and incorporation into OBs [44]. The source of the ODV envelope is unclear. A number of ODV envelope proteins have been found to associate with intranuclear microvesicle structures that appear in the nucleus during infection, thereby suggesting they may be the source of the envelope [27–29].

Occlusion bodies accumulate until the terminal stages of infection when the insect liquefies and the occlusion bodies are released into the environment. Liquefaction appears to be caused by the production of both virus-encoded chitinase and cathepsin genes [59–61] and may also involve the *fp25k* product [62]. Deletion of the cathepsin gene from *Bombyx mori* NPV reduced recombinant protein degradation in virus-infected insects, presumably because of the reduction in proteinase activity [63]. Just prior to death many Lepidopteran species crawl to the top of the vegetation on which they were feeding and hang from this elevated position, facilitating dissemination of the virus as the cadaver decomposes [64]. This enhanced locomotory activity (ELA) may well be virus-induced, since insects infected with a *B. mori* NPV mutant lacking the protein tyrosine phosphatase gene showed dramatically reduced ELA before death after about 5 days [65]. However, removal of ecdysteroid glucosyltransferase gene (*egt*) from a baculovirus was found to reduce vertical movement of virus-infected insects on plants [66]. Occlusion bodies serve as survival vehicles for the virus when outside the insect host, as well as acting as dispersal agents between individual insects. They may also protect the virus against proteolytic decay during the end stages of infection [41].

5 Baculovirus Gene Expression and Replication

The complete sequence of AcMNPV clone 6 has been determined. The original analysis of the 133,894 bp genome suggested that the virus encodes 154 methionine-initiated, potentially expressed open reading frames (ORFs) of 150 nucleotides or more [67]. However, resequencing of various regions of the genome and comparison with other virus isolates has corrected a number of errors and suggests that AcMNPV may only encode 150 genes. Other baculoviruses have also been sequenced. An up-to-date summary of completed genomes is available from the National Center for Biotechnology Information (NCBI). Baculovirus gene expression is divided into four temporal phases: immediate-early, delayed-early, late, and very late, although the first two are often considered as one. Immediate-early genes are distinguished from delayed-early by their expression in the absence of *de novo* protein synthesis. Expression of delayed-early genes, however, appears to be dependent on the presence of immediate-early gene products. Transcription of late genes occurs concurrently with the onset of viral DNA replication at about 6 h pi. Very late gene transcription begins at about 20 h pi and involves high levels of expression from the polyhedrin and *p10* promoters, two proteins involved in the occlusion of virions. While levels of late gene mRNA transcripts decrease at very late times in infection, very late polyhedrin and p10 gene transcript levels continue to remain high [68].

Analysis of the AcMNPV genome has revealed that RNA transcripts are not clustered according to their temporal expression. Instead, early and late genes are found distributed throughout the genome. All AcMNPV RNAs are both 5′ methyl capped and 3′ polyadenylated [69, 70]. Only one transcript, which is that of the immediate-early gene *ie-1*, is known to be spliced [71]. However, transcriptional units involving overlapping RNAs have been identified in the AcMNPV genome, which may provide an alternative means of introducing a variety of expression [72, 73]. Overlapping transcripts composed of early and late RNAs with a common 3′ end have been identified in a number of regions [72, 73]. The *Hin*dIII-K fragment of the AcMNPV genome produces five overlapping RNAs, two immediate-early, one delayed-early, and two late gene transcripts, transcribed in the same direction and terminating at a common 3′ end [73]. This arrangement has been implicated in the temporal regulation of these genes. It has been suggested that the longer, later 5′ extended transcripts serve to repress transcription of earlier genes, located downstream, probably by means of promoter occlusion, as well as acting as mRNAs for late viral products [73].

The polyhedrin and p10 genes are also transcribed as several overlapping RNAs. In the case of these two genes, however, transcripts have common 5' ends, with longer RNAs being derived from read through of termination signals at the 3′ end of the smaller RNAs [72–74]. Four overlapping transcripts were mapped to the p10 gene region and were found to comprise two sets: a late phase pair of transcripts (1100 and 1500 bases) sharing a common 5′ end that are most abundant at 12 h pi and a very late phase pair (750 and 2500 bases) that also have a common 5′ end and are most abundant at 24 h pi [74]. Promoter occlusion may also be operating in this region. Synthesis of earlier transcripts may prevent RNA polymerase initiation at the *p10* promoter located downstream. At later times, when transcription from upstream promoters has ceased, RNA polymerase may be able to initiate at the downstream *p10* promoter [74].

5.1 Early Gene Expression

Baculovirus early genes are transcribed before the onset of viral DNA replication. Expression of many early genes begins immediately after cell infection, with some transcripts, for example the anti-apoptotic p35 gene early mRNAs, appearing within the first 2 h [75]. Genes in this phase of the virus lifecycle are transcribed by the host RNA polymerase II, as demonstrated by the fact that early transcription is sensitive to α-amanitin [76], a compound that binds to and inactivates the large subunit of RNA polymerase II.

5.1.1 Promoter Elements

Most early baculovirus promoters contain a TATA element which, as well as regulating the rate of transcription initiation, also establishes

the position of the RNA start site 25–30 bases downstream [71, 77–79]. An initiator motif (ATCA(G/T)T(C/T)), which overlaps the RNA start site, has also been identified in many early promoters. The most conserved of these is the CAGT motif that has been found to contribute to basal promoter activity, as well as being sufficient in determining the position of the RNA start site in the absence of a TATA element [79–82]. In composite promoters, those containing both CAGT and TATA motifs, cooperation is thought to occur between the two to stabilize host transcription machinery or enhance recruitment of required factors [83].

The early promoter of *dnapol*, which encodes the viral DNA polymerase, does not contain a TATA or CAGT motif. Early transcription from this promoter initiates from multiple sites including the sequence CGTGC [84]. This sequence is also found at the start of *p143* [85]. The significance of this unusual promoter motif is unknown, although it is thought that these promoters may be more responsive to viral transactivators during infection [83].

Another *cis*-acting element identified in early promoters is a downstream activating region (DAR). A DAR has been identified in the 5′ noncoding regions of the immediate-early *ie-1* gene [84]. This element, located between positions +11 and +24, is necessary for optimal expression of *ie-1* early in infection [81]. The core DAR sequence (A/T)CACNG has also been identified in the 5′ noncoding region of the envelope glycoprotein encoding gene *gp64*, and has been found to stimulate the rate of early *gp64* transcription [81, 86].

Many early baculovirus promoters have also been found to possess an upstream activating region (UAR), consisting of one or more *cis*-acting DNA elements that affect the level of transcription, but not the position of the RNA start site [78, 79, 87]. The *ie-1* UAR has been found to increase promoter activity twofold early in Sf-21 cell infection [81], while the *p35* UAR was shown to be responsible for a 10- to 15-fold enhancement of basal transcription [78]. A number of distinct UAR elements have been identified. The first consists of GC-rich sequences and, as such, is termed the GC motif. GC motifs have been found in the UARs of a number of early baculovirus promoters including those of *p35* and *39K*, a gene encoding a protein (pp31) associated with the virogenic stroma [78, 79, 88]. The CGT motif consists of the consensus sequence A(A/T)CGT(G/T) and has been identified in the UARs of *p35*, *39K* and the helicase encoding gene *p143* [78, 79, 85]. A third UAR motif, referred to as the GATA element, has also been identified in the early *gp64* promoter and in that of the immediate-early *pe-38* [86, 89].

5.1.2 Transcriptional Enhancers

The AcMNPV genome contains homologous regions (*hrs*), rich in *Eco*RI sites, distributed throughout the genome [90]. Eight of these regions (*hr*1, *hr*1a, *hr*2, *hr*3, *hr*4a, *hr*4b, *hr*4c, *hr*5) have been

identified and were found to consist of two to eight copies of a 28 bp imperfect palindromic repeat (28-mer) bisected by an *Eco*RI site and flanked on each side by direct repeats of about 20 bp [67, 91]. Several early viral promoters, including those of *39K*, the immediate-early gene *ie-2* (formerly *ie-n*), *p143* and *p35*, have been shown to be stimulated by *cis*-linkage to *hrs* [80, 85, 87, 92–94]. Promoter enhancement by *hrs* occurs in a position- and orientation-independent manner [93] and this enhancement is further augmented by the immediate-early IE-1 protein [91], a viral transactivator which has been found to bind to *hr* sequences [94–96]. The *hr* 28-mer is the minimal sequence required for IE-1 mediated promoter enhancement [92–94]. The IE-1 protein binds to the 28-mer as a dimer, interacting with the two palindromic half-sites, both of which are required for *hr* enhancer activity [93–96]. Oligomerization of IE-1 is thought to occur in the cytoplasm, before localization to the nucleus, binding to *hr* sequences, and subsequent enhancement of promoter activity through interaction with components of the basal transcription complex [95, 97].

5.1.3 Transactivational Regulators

A number of transactivational regulators of baculovirus early gene promoters have been identified. The immediate-early gene, *ie-1,* is thought to be the principal transregulator of early baculovirus expression and was originally identified due to its *trans*-acting regulatory role in *39K* expression [92]. As well as stimulating expression of genes such as *p35*, *p143*, and *39K* [85, 87, 92, 98], IE-1 is capable of stimulating its own promoter [99]. The N-terminal region of the 582 residue IE-1 protein has been found to contain a transactivation domain, while the C-terminal of the protein contains a DNA binding domain [95]. A small basic domain between residues 537 and 538 has been identified as a nuclear localization signal, which functions upon dimerization of IE-1 [97]. Transcripts of *ie-1* give rise to both spliced and unspliced RNAs. Unspliced transcripts encode IE-1 itself, while spliced transcripts encode another immediate-early transregulator, IE-0, identical to IE-1 except for 54 additional amino acids at its N-terminus [71, 99]. While IE-0 is expressed only during the early phase of infection, IE-1 RNA is expressed in both the early and late phases [71]. Transient expression assays have shown IE-1 to have a negative regulatory effect on *ie-0* promoter expression, while IE-0 transactivates the *ie-1* promoter [99]. Deletion of *ie-1/ie-0* from the virus genome using an *Escherichia coli*-based system prevented virus replication in insect cells, although restoration of the mutant with either gene largely restored production of infectious virus progeny [100].

The transcriptional regulator IE-2 indirectly stimulates expression from promoters dependent on IE-1 for transactivation, by increasing transcription from the *ie-1* promoter. The protein has been shown to be capable of enhancing IE-1 transactivation of both

the *p143* and *39K* promoters [85, 101]. In addition to enhancing IE-1 expression, IE-2 stimulates expression of IE-0 in transient assays, as well as auto-regulating its own expression [80]. Like IE-0 expression, IE-2 regulation has been shown to be down-regulated by IE-1 [102]. Another transactivational regulator, encoded by *orf121*, has been shown to stimulate the *ie-1* promoter in a similar manner to IE-2 in transient assays [103].

The immediate-early gene *pe-38* encodes a 38 kDa protein, which also acts as an early transregulator. The p143 gene promoter is transactivated by PE-38, and this transactivation has been found to be augmented by IE-2 [85]. However, the delayed-early *39K* promoter was not stimulated by PE-38 [85], suggesting that PE-38 has a restricted transactivation range compared to IE-1, which is capable of stimulating both promoters [85, 92].

Transcripts of *ie-0*, *ie-2* and *pe-38* are expressed during the early phase of infection, while *ie-1* RNAs are expressed during the early and late phases. The fact that IE-1 down-regulates expression of both IE-0 and IE-2 in transient assays suggests that it may function to shut off immediate-early gene expression during the late phase of infection [99, 102], while IE-1 stimulation of its own promoter suggests that it positively regulates its own expression during infection [99]. Baculovirus *pe-38* is transactivated by IE-1 when both are transfected into mammalian BHK-21 cells [104].

5.2 Baculovirus and Apoptosis

As one of the first viral families found to be capable of regulating host apoptotic pathways, the baculoviruses have become important tools in the study of apoptosis [105, 106]. Apoptosis may have evolved to remove unwanted cells in the development of an organism but has been adapted as an antiviral defense mechanism. Members of the *Baculoviridae* encode a number of important apoptotic suppressors. The study of the mechanism of action of these proteins in baculovirus-infected insect cells has revealed important information about conserved points in the cell death pathway. Additional information about the use of baculoviruses in the study of apoptosis can be found in Chapter 25 of this book.

AcMNPV infection of Sf-21 cells induces apoptosis, thereby resulting in the activation of the novel insect caspase SF-caspase-1, an effector caspase found to have sequence similarity to human caspase-3,-6, and-7 [107]. Expression of the AcMNPV anti-apoptotic p35 gene blocks apoptosis allowing replication of the virus to proceed [105]. The specific factors that activate the cell death pathway in AcMNPV-infected Sf-21 cells are unclear. While budded virus binding alone is not sufficient to induce apoptosis, transient expression of the IE-1 protein has been found to induce cell death in Sf-21 cells [108]. Cell death induced by IE-1 is further enhanced by *pe-38* in transient assays, although the precise mechanism by which this augmentation takes place remains unclear [109].

Although some induction of apoptosis occurs upon IE-1 expression, viral DNA replication is required for the full apoptotic response [110, 111]. This is supported by the finding that the timing of DNA synthesis coincides with activation of SF-caspase-1 and the occurrence of the first morphological signs of apoptosis, such as cell membrane blebbing [110]. It is possible that DNA synthesis induces apoptosis indirectly by promoting the onset of late gene expression. Alternatively, viral DNA replication may activate apoptosis directly by damaging cellular DNA or disturbing the insect cell cycle.

The baculovirus anti-apoptotic p35 gene was first identified during characterization of an AcMNPV spontaneous mutant. The mutant, termed the annihilator (vAcAnh), was found to cause premature death in *S. frugiperda* (Sf-21) cells, but not in *T. ni* (Tn-368) cells [105]. Infection of Sf-21 cells with wild type AcMNPV causes transient plasma membrane blebbing at approximately 12 h pi [105]. A similar effect was observed with vAcAnh infection of Sf-21 cells. However, while this blebbing disappeared in the wild type infection, it was found to intensify with the mutant infection, thereby resulting in disintegration of cells into apoptotic bodies [105]. Cell blebbing was not observed in *T. ni* cells infected with either the wild type virus or vAcAnh, allowing amplification of the mutant in this cell line [105]. Annihilator mutant-infected Sf-21 cells also exhibited a number of other features of apoptosis, including nuclear condensation, intact mitochondria retention until late in the apoptotic process, and internucleosomal cleavage of cellular DNA beginning between 6 and 12 h pi [105]. A deletion in the p35 gene, located in the *Eco*RI-S fragment of the AcMNPV genome, was subsequently identified as being responsible for the annihilator mutant phenotype [105]. The p35 gene is transcribed from a promoter containing both early and late start sites, although it is predominantly transcribed as an early gene, with transcripts detectable within the first 2 h of infection [75, 78, 87]. The gene encodes a 299 amino acid protein with no recognizable sequence motifs.

Another class of anti-apoptotic genes, the inhibitor of apoptosis (*iap*) genes, has also been identified. Baculovirus IAP proteins block apoptosis in Sf-21 cells induced by a number of different stimuli other than baculoviral infection, including treatment with apoptosis inducing agents such as actinomycin D, cycloheximide, tumor necrosis factor α and UV light [112]. A *Cydia pomonella* granulovirus (CpGV) iap gene product, Cp-IAP-3, was the first IAP protein to be identified and confirmed to have anti-apoptotic activity. This Cp-iap-3 gene was initially identified during a genetic screen for genes that could complement the absence of p35 in annihilator mutant-infected Sf-21 cells. Cp-IAP-3 is expressed both early and late in infection from distinct transcription start sites and has been found to localize in the cytoplasm, with no

IAP-3 detectable in the nuclei of infected cells [113]. Five iap genes have been recognized: iap-1 is found in Group I NPVs, iap-2 occurs in both Group I and II NPVs, iap-3 is encoded by Group I, II, GVs and hymenopteran NPVs, whereas iap-4 is present in a few Group I and II NPVs and iap-5 occurring solely in GVs [114].

A characteristic of all members of the IAP family is the presence of 1–3 copies of an imperfect 70 amino acid repeat called a Baculovirus IAP Repeat (BIR) at the N-terminus of the protein [106]. Baculoviral IAPs, and several cellular IAPs, also contain a carboxy terminal RING finger (zinc-like-finger) motif. Both BIR and RING domains are thought to be involved in protein–protein interactions and are essential for inhibition of apoptosis, with the BIRs having been implicated directly in the binding and inhibition of caspases [115]. The mechanism by which baculovirus IAPs block apoptosis is distinct from that of P35. While P35 is capable of directly interacting with and inhibiting the active caspase, IAP acts upstream of this by inhibiting maturation of the procaspase [116].

5.3 Baculovirus Replication

The homologous regions (*hrs*), identified as enhancers of early gene expression, have also been proposed as origins of viral DNA replication [90]. Evidence for this role was obtained through assays of transient replication, which have shown plasmids containing *hrs* to be capable of AcMNPV dependent replication when these were used to transfect Sf-21 cells [117]. As with early promoter enhancement, a single 28-mer is sufficient to support plasmid replication [94]. However, deletion mutagenesis of *hr5*, which contains six palindromes, revealed the efficiency of replication from individual *hrs* to be dependent on the number of palindromes presents [118]. An AcMNPV non-*hr* containing origin has also been identified in the *Hin*dIII-K fragment of the genome [117]. A circular topology has been found to be a requirement for replication of origin-containing plasmids, suggesting the mechanism of baculovirus DNA replication involves a theta or rolling circle intermediate [117].

Six genes, encoding P143 (DNA helicase), DNA polymerase [119], IE-1, late expression factor-1 (LEF-1) (primase), LEF-2 (primase associated protein) [120], and LEF-3, (single-stranded DNA binding protein) [121, 122] have been found to be essential for transient DNA replication [123]. In addition, *lef-11* was reported to be essential for AcMNPV replication in Sf-9 cells [124]. DNA-independent ATPase activity has been associated with the DNA helicase of *Trichoplusia ni* granulovirus [125]. Deletion of the DNA polymerase gene from the virus genome abrogates virus replication [126]. LEF-2 is a capsid protein not required for initiation of DNA replication but is necessary for amplification [127].

Genes encoding P35, IE-2, PE-38 and LEF-7 are thought to play a stimulatory role in DNA replication [123, 128]. The stimulatory effect of the anti-apoptotic *p35* gene in transient assays is

thought to be largely due to its role in preventing premature cell death, although there is evidence suggesting that P35 may also be involved in early gene regulation [98]. The *pe-38* gene product has been seen to play a role in the activation of expression of the baculovirus helicase homolog P143, while IE-2 stimulates *pe-38* and *ie-1* expression [85, 129]. Stimulation of viral DNA replication by LEF-7 was observed [128] and this protein has been found to contain two single stranded binding protein (SSB) motifs.

Baculovirus DNA replication is associated with distinct foci in the nuclei of infected cells [130]. It was found that IE-2, LEF-3 and an additional protein thought to play a role in AcMNPV replication, termed DNA binding protein (DBP), colocalized with centers of viral DNA replication within the nucleus [130]. The importance of LEF-3 in DNA replication is further underlined by its involvement with viral helicase and polymerase. The protein interacts with P143 and is required for nuclear localization of the helicase [131, 132]. It has also been found to play a role in enhancing the strand displacement activity of DNA polymerase [119]. Interaction between LEF-1 and LEF-2 has been observed, and is thought to be required for DNA replication [133, 134]. The IE-1, LEF-3 and P143 products interact with DNA in vivo, as demonstrated by formaldehyde cross linking studies [135]. Deletion of very late factor 1 (vlf1) reduces DNA replication to a third of normal levels and no budded virus is produced [136]. Virus DNA levels double every 1.7 h from 6 h pi until about 20 h pi [137]. By this point each cell contains about 84,000 genomes.

5.4 Late and Very Late Gene Expression

5.4.1 Viral RNA Polymerase

The RNA polymerase responsible for transcription of late and very late baculovirus genes is encoded by the virus itself [138]. This viral polymerase is α-amanitin-resistant and unable to transcribe from early gene promoters [139]. The products of four lef genes, *lef-8*, *lef-4*, *lef-9* and *p47*, have been identified as components of the AcMNPV RNA polymerase [138]. The LEF-4 has guanylytransferase activity [140] and RNA 5′ triphosphatase and nucleoside triphosphatase activity [141]. Genes encoding each of the RNA polymerase subunits have been shown to be necessary for late and very late gene expression in transient expression assays [128, 142, 143]. Studies on temperature sensitive mutants had previously identified similar roles for p47 and LEF-4 in late gene transcription, suggesting them to be members of the same protein complex [144]. Pairwise interactions between LEF-9 and P47, LEF-4 and P47, and LEF-8 and P47 have been demonstrated, but interactions between LEF-4 and LEF-8 do not occur unless P47 is present [145].

The viral RNA polymerase is thought to carry out both 5′ methyl capping and 3′ polyadenylation of late and very late transcripts [140, 141, 146]. The RNA triphosphate cleaves the 5′-triphosphate from primary transcript termini, producing the guanylyltransferase substrate. The final stage of the reaction

requires RNA methyltransferase to catalyze the transfer of a methyl group to the guanosine cap. The protein responsible for this stage of the process remains to be confirmed, although it has been suggested that a host cell enzyme could carry out this step [138, 141]. LEF-4 has also been found to possess an ATPase activity, although the function of this activity is unknown [146]. Both LEF-8 and LEF-9 contain conserved motifs present in the large subunits of other DNA-directed RNA polymerases [146, 147]. The conserved motif of LEF-8 had been proposed as a putative catalytic site of the enzyme [146]; however, *lef-8* mutagenesis studies have revealed that both termini of the protein are essential for its function [148]. In addition, a host cell protein, designated polyhedrin promoter binding protein (PPBP), has been identified that binds to very late promoters and is required for expression of these genes [149, 150]. The protein has both double stranded and single stranded binding activities and may act as an initiator binding protein similar to the TATA-binding protein (TBP) required for transcription initiation by eukaryotic RNA polymerases I, II and III [150].

5.4.2 Promoter Elements

Baculovirus late and very late transcripts initiate at the central A of a conserved (A/G)TAAG sequence, usually ATAAG for abundant transcripts, which is essential for promoter activity [6, 7, 151]. Analysis of the *gp64* promoter region, which contains two active and three inactive TAAG motifs, revealed that sequences immediately surrounding the initiation site, rather than its position, determine its use as a late gene promoter [152]. Mutations in sequences surrounding TAAG have been shown to reduce transcription at the level of transcription initiation [153]. However, the TAAG motif and its surrounding sequences are not involved in mediating the difference in temporal regulation between late and very late transcription. The very late polyhedrin and p10 genes are abundantly expressed at very late times in the infection. Despite having little homology, the 5′ leader sequences of both promoters are extremely A+T rich and contain sequences necessary for the burst in expression observed very late in infection [153–156]. Mutations upstream of the TAAG motifs of both very late promoters exert a mild effect on expression [154, 157]. Thus, the polyhedrin promoter consists of a 49 bp 5′ untranslated region and 20 bp upstream of the transcription start site [154], while the *p10* promoter consists of a 70 bp 5′ untranslated region and 30 bp upstream of the transcription start site [155, 157]. Polyhedrin and *p10* promoters appear to be regulated differently, with *p10* expression occurring earlier in infection and at lower levels than that of polyhedrin [158].

5.4.3 Regulation of Late and Very Late Gene Expression

Nineteen Late Expression Factor (*lef*) genes of AcMNPV, which are necessary and sufficient for transient expression from both late and very late viral promoters in Sf-21 cells, have been identified [143, 159–161]. A subset of these genes is involved in plasmid

DNA replication, while another four are thought to be subunits of the viral RNA polymerase, as outlined above. The remaining *lefs* (*lef-5, lef-6, lef-10, 39K, lef-12*) are thought to function in late promoter recognition or stabilization of late transcripts [128]. In addition to its role in plasmid DNA replication, *ie-1* is thought to have a direct effect on expression from the very late polyhedrin promoter [162]. Further genes involved in transient late gene expression have been identified [161]. One of these genes, termed *orf41*, was found to be necessary for late gene expression, while *orf69* was found to play a stimulatory role [161]. LEF-12 is dispensable for virus replication [163]. It was suggested that IE0 could be regarded as the 20th LEF since it can replace IE1 in a transient replication assay [164].

The FP25K protein, a late gene product thought to be a structural component of the nucleocapsid, enhances the rate of transcription from the very late polyhedrin promoter, but not that of the *p10* promoter [165]. The effect of this protein on transcription of only one of these very late hyperexpressed genes is consistent with the differential regulation observed for the two promoters [158].

The Very Late Expression Factor-1 (*vlf-1*), is specific for regulation of very late transcripts [166]. The encoded protein, VLF-1, is required for high level expression from the polyhedrin and *p10* promoters and is thought to exert its effect by interacting with the burst sequences located between the transcriptional and translational start sites of each promoter [167]. Although *vlf-1* is mainly transcribed at late times in infection, the stability of the protein allows it to remain at high levels throughout the very late phase [168, 169]. Accelerated production of VLF-1 results in premature polyhedrin synthesis, showing that the timing of VLF-1 expression is important in very late gene transcription [169].

VLF-1 has also been found to play a role in BV production, possibly as a resolvase or topoisomerase to produce monomeric viral genomes from concatemeric products of the DNA replication process [168]. The predicted sequence of the 44.4 kDa VLF-1 protein shows similarity to the sequences of a large class of resolvases and integrases found in *Saccharomyces cerevisiae*, prokaryotes and phages [166]. Viruses carrying *vlf-1* null mutations have been found to be either nonviable or so defective in BV production that they were extremely difficult to propagate, thereby indicating an essential role for the protein in baculovirus replication [168]. The level of VLF-1 protein required for activation of very late promoter burst sequences is thought to be much higher than is required for the protein's role in BV production [169]. Overexpressed and purified VLF1 added to transcription assays containing baculovirus RNA polymerase stimulated transcription of the polyhedrin gene promoter, but not 39K [170].

Serial passage of Nucleopolyhedroviruses through cultured cell lines results in the appearance of a spontaneous mutant termed the 'few polyhedra' (FP) mutant [171, 172]. With continued passage this FP phenotype becomes dominant [173]. Fourteen passages of *Trichoplusia ni* (Tn)MNPV in *T. ni* cells was found to result in a purely FP mutant population [173]. The characteristics most commonly associated with the FP phenotype are a reduced number of polyhedra per cell compared to the wild type, occlusions containing no virions or virions of altered morphology, altered intranuclear envelopment, and the production of more BV than cells infected with the wild type [172–174].

A common feature of many AcMNPV and *Galleria mellonella* (Gm)MNPV FP mutants is insertion of DNA sequences (0.8–2.8 kb), homologous to moderately repetitive host DNA, into a region of the genome encoding a 25 kDa protein (i.e., the FP25K protein). Subsequent studies correlated AcMNPV FP mutations to large insertions of host cell DNA or deletions of viral DNA, detectable by restriction endonuclease (RE) analysis, in this region of the genome [175, 176]. Targeted mutation of AcMNPV *fp25k* confirmed alterations in this gene to be sufficient to cause the complex characteristics of the FP phenotype, including reduced virion occlusion, altered intranuclear envelopment and enhanced BV production [177].

A late gene product, the FP25K protein, is highly conserved among members of the Nucleopolyhedroviruses, with the last 19–26 C-terminal amino acids the only region lacking significant conservation among sequenced *fp25k* genes [178]. Although identified as a structural protein of the nucleocapsids of BV and ODV [179], a large fraction of the protein remains associated with amorphous cytoplasmic bodies throughout infection [179].

Mutations in *fp25k* alter the apparent expression and/or accumulation of several viral proteins. Rates of both polyhedrin biosynthesis and nuclear localization are reduced in cells infected with *fp25k* mutants [180]. The effect of FP25K on polyhedrin biosynthesis was found to occur at the level of transcription, with wild type FP25K found to enhance expression from the polyhedrin promoter [165]. While the rate of polyhedrin transcription in *fp25k* mutants was reduced, *p10* RNA levels were unaffected, suggesting that the reduction in polyhedrin RNA does not reflect a general effect on very late gene expression [165]. Acquisition of mutations within *fp25k* in baculovirus expression vectors could, therefore, seriously affect recombinant protein production. This might occur if recombinant virus is produced and amplified in *T. ni* cell lines. Thus, most commercial baculovirus expression systems guard against using *T. ni* cells for virus production and amplification, recommending these cells for protein production only.

6 Baculoviruses as Expression Vectors

Two features of baculoviruses underpin their use as expression vectors. The first is that the very late polyhedrin and p10 genes are dispensable for virus replication in cell culture and in insects if the budded virus is delivered to the hemocoel of the larval host [180–182]. The second is that both of these virus gene promoters are very strong and if coupled with a foreign gene coding region can enable the production of large amounts of recombinant protein in insect cells. Recent studies to elucidate the nature of the very late virus gene promoters have also enabled expression vectors to be derived that contain multiple copies of the polyhedrin and p10 promoters so that several recombinant proteins can be produced simultaneously in virus-infected cells [183–185]. These vectors have been of particular use in assembling structures in insect cells that are composed of more than one protein [186]. Insect cells are also competent in accomplishing many of the post translational processes required when producing proteins from eukaryotic cells, thereby producing biologically active products.

The original method for producing recombinant baculoviruses required replacing the native polyhedrin gene with the heterologous coding sequences, thus deriving a polyhedrin-negative virus. This virus had to be identified by visual selection of plaques lacking polyhedra in a standard virus titration. While moderately difficult to the experienced user, it often proved impossible for the novice. Fortunately, this problem has been solved by a wide variety of newer methods that enable modification of the virus genome to be done more easily. Currently, automated systems for making recombinant baculoviruses are being devised that promise to facilitate the simultaneous production of dozens, if not hundreds, of expression vectors. Baculoviruses have also become a useful tool for introducing foreign genes into human cells, where the lack of virus amplification means that there need be no concerns over biosafety of the gene delivery vector [187].

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