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The Baculovirus Expression Vector System: Regulation of the Polyhedrin Gene Promoter

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The baculovirus expression vector system (BEVS) is very commonly used for high level synthesis of heterologous proteins in insect cells and larvae. In BEVS the foreign gene is transcribed by the hyperactive viral polyhedrin gene promoter activated very late during *Ac*NPV (*Autographa californica* nuclear polyhedrosis virus) infection. This review, while briefly attempting to introduce the trivialities of this system, focuses on the regulation of the polyhedrin gene promoter which is of special interest particularly because of its strength and temporal profile of activation.

Key Words: Baculovirus expression Vector system, Heterologous protein, Insect cells, AcNPV, Polyhedrin gene promoter

Introduction

The expression of heterologous genes in insect cells using the Baculovirus Expression Vector System (BEVS) has become a popular and powerful tool for the purification and analysis of a wide variety of eukaryotic and prokaryotic proteins (for reviews see Luckow 1991, Jarvis & Summers 1992, O'Reilly et al. 1992).

Baculovirus-infected insect cells perform many of the post-translational and chemical modifications and proteolytic processing events seen in higher eukaryotes (O'Reilly et al. 1992) such as glycosylation, fatty acid acylation, amino-terminal acetylation, carboxy-terminal α-amidation, myristylation, and phosphorylation. In most cases, the recombinant proteins are targeted to their natural locations within the cell. Proteins containing signal peptides are recognized and properly cleaved before insertion into cellular membranes or secretion from the cell. Also, hetero- and homo-oligomeric assembly have been demonstrated for a wide variety of proteins in baculovirus-infected cells. This, along with the high expression levels that can be achieved, accounts for the immense popularity of BEVS, which is evident from the fact that this system has been used in over 1,000 different laboratories throughout the world with more than 500 genes from viruses, bacteria, fungi, plants, and animals having been expressed so far in both insect cells and larvae. The simultaneous, but contrasting levels of synthesis of two different reporter proteins (βhCG and luciferase) in BEVS using a dual expression recombinant virus has been reported (Jha et al. 1992, Hasnain et al. 1994). This difference in expression could be attributed to a number of factors such as relative promoter strength, differences in the

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translational efficiency of the two mRNAs due to features intrinsic to the gene (Ranjan & Hasnain 1994), and/or post-translational modifications and secretory load (Nakhai et al. 1991, Sridhar & Hasnain 1993, Sridhar et al. 1993).

Baculovirus Structure and Molecular Biology

Baculoviruses are a diverse group of insect viruses with rod-shaped capsids usually 40-50nm in diameter and 200-400nm in length (Harrap 1972b). Within the capsid, the DNA is condensed into a nucleoprotein structure known as the *core*. The DNA genome of a baculovirus is double-stranded, covalently closed, and circular (Summers & Anderson 1972). The DNA of the prototype baculovirus, the *Autographa californica* nuclear polyhderosis virus (AcNPV), is approximately 130 kbp.

Nucleocapsids are made in the nucleus of infected cells and are enveloped by one of two processes:

(i) Nucleocapsids can bud through the plasma membrane into the extracellular fluid. This *budded virus* (BV) acquires a loosely fitting membrane envelope.

(ii) Nucleocapsids may also acquire a "de-novo" envelope within the nucleus (Stoltz et al. 1973). Such virions are then occluded within a crystalline protein matrix within the nucleus, and such virus particles are called *occluded virus* (OV). The protein making up the crystalline protein matrix is polyhedrin and the polyhedral occlusion bodies are known as *polyhedra* (Harrap 1972a).

The viral infection cycle can be divided into three phases—early, late, and very late.

Early phase: The first 6 hr of infection constitutes the early phase and it is during this period that the cell is reprogrammed for virus replication. Infection in cell culture is mediated by BVs which enter the cells by adsorptive endocytosis. Nucleocapsids migrate to the nucleus where the nucleoprotein core is released by the uncoating of the capsid within 1 hr pos infection (hpi) (Granados & Williams 1986). Infected cells undergo cytoplasmic and nuclear changes during this period-cytoskeletal rearrangements occur, the host chromatin disperses, and the nucleus becomes enlarged.

Late phase: This phase extends from 6 to 20hpi. During this period viral DNA replication, late gene expression, and BV production take place (Knudson & Harrap 1976).

Very late phase: This phase begins around 20hpi and is characterized by the accumulation of polyhedra within the nucleus (Fraser 1986). Also, large arrays of fibrous material, composed of a 10-kDa protein, p10, accumulate in the nucleus. This fibrous material may play a role in the lysis of host cells (Williams et al. 1989) which usually begins around 60hpi and is complete by about 72hpi.

Baculovirus Gene Regulation

Baculovirus genes are transcribed in three phases—early, late, and very late—in a regulated cascade, with gene products of one temporal class trans-activating (directly or indirectly) transcription of the genes of the next temporal class (for a review see Blissard & Rohrmann 1990).

Early gene transcription: Early genes have been defined as those genes that are transcribed in the absence of any viral gene expression. Expression of several early genes is dependent on the product of the *ie-1* gene, IE-1, which is believed to enter the cell as a component of the virion (Guarino & Summers 1987). Induction of early promoters by IE-1 can be enhanced by the presence of the *ie-n* gene product and homologous region (hr) sequences, the latter

being repeats of apalindromic sequence present at five locations in the AcNPV genome (Guarino & Summers 1987). Early gene transcription is mediated by host RNA polymerase II (sensitive to α-amanitin) and no viral gene expression is required.

Late and very late gene transcription: Late and very late gene transcription is insensitive to α-amanitin (Huh & Weaver 1990b) and involves a novel or modified RNA polymerase and virus-specific factor(s). Late and very late gene transcription is dependent on early gene expression and on DNA replication—both cycloheximide and aphidicolin block transcription of late and very late genes (Huh & Weaver 1990a); uninfected *Spodoptera frugiperda* cells do not support late or very late gene expression when transfected with a reporter plasmid (Hoopes & Rohrmann 1991). However, the basis for this dependence of late gene transcription on DNA replication is not known.

Late and very late promoters can be distinguished by their relative activities during the late and very late phases. Very late promoters display low activity during the late phase (6-20hpi) but become highly active during the very late phase (20-72hpi). On the other hand, late genes are more active during the late phase.

The primary determinant of late and very late promoter activity is the pentanucleotide (A/G)TAAG, which is located at the transcription start point of all known late and very late genes (Possee & Howard 1990, O'Reilly et al. 1992).

Five genes, designated *lef*-1, *lef*-2, *lef*-3, *lef*-4, and *lef*-5 (for late expression factor), are essential but not sufficient for late and very late gene expression (Li et al. 1993, Passarelli & Miller, 1993a, 1993b, 1993c). However, it is not known whether the *lef* gene products act at the level of replication, transcription, or translation.

The Polyhedrin Gene Promoter of AcNPV

The *polh* promoter has been the "workhorse" promoter of BEVS. It is a very strong promoter that is expressed very late in infection and is responsible for the synthesis of polyhedrin protein. The *polh* promoter of AcNPV has been extensively characterized by deletion and linker-scan mutation analyses. The transcription start point is at −50 relative to the translational start site (designated as +1) and lies within a highly conserved octanucleotide sequence TAAGTATT. The essential polyhedrin promoter has been defined (by experiments described below) as a 69 bp stretch (−1 to −69) with the TAAGTATT motif being absolutely essential for transcription initiation.

It was shown by Matsuura et al. (1987) that the full 5′ leader sequence of the *polh* gene (−1 to −50) was required for maximum expression of foreign genes in the baculovirus system. In order to determine the effect of the integrity of the leader sequence on the expression of a reporter gene (the hemagglutinin gene of influenza A virus in this case), Matsuura et al. 1987 made a series of recombinant viruses with variable lengths of the leader sequence. Sequential deletions into the leader sequence upstream from the *polh* ATG adversely affected reporter gene expression.

Possee and Howard (1987) made recombinant viruses with deletions in the promoter which progressively removed sequences upstream to the transcription start point. The effect of these deletions on *polh* promoter activity was judged by expression of the beta-galactosidase gene inserted in lieu of the polyhedrin coding sequences. A sequence 69 nucleotides upstream to the *polh* translational initiation codon was sufficient for maximum promoter activity. Likewise, insertions of varying sizes −8, 95, and 785 nucleotides (the latter representing the full complement of the CAT

gene)—into the EcoRV site upstream to the *polh* promoter (−92) did not affect reporter gene expression. This data, coupled with that of Matsuura et al. (1987), suggests that the polyhedrin promoter resides within a 69 bp fragment (−1 to −69). It is pertinent to mention here that in all of these experiments the ORFs upstream to the *polh* gene were intact. Therefore, the effect of such upstream sequence elements on *polh* promoter-driven transcription cannot be excluded.

Ooi et al. (1989) constructed a series of recombinant baculoviruses containing linker-substituted *polh* promoters attached to a reporter gene encoding chloramphenicol acetyl transferase (CAT) and tested for expression of the gene. The major determinant for promoter activity was narrowed down to the octanucleotide motif, TAAGTATT, at the transcription start point. Mutations within TAAGTATT blocked initiation of transcription from this site and resulted in a 2000-fold decrease in CAT activity. Mutations downstream from TAAGTATT and within the region specifying the untranslated RNA leader diminished transcription initiation and decreased CAT activity 2- to 20-fold. The half-lives of CAT RNAs were not affected by mutations in the untranslated RNA leader region; nuclear run-on analysis showed that these mutations decreased the rate of transcription initiation. Thus, the low steady-state RNA levels in such linker-scan mutants are due to less efficient transcriptional initiation, not an alteration in RNA turnover rates. Transcription initiation thus appears to be the major means of polyhedrin gene regulation.

Ooi et al. (1989) proposed a model wherein the (A/G)TAAG sequence serves as an essential component for the recognition of both late and very late promoters by the virus-induced RNA polymerase activity. Additional factors (binding to the cis-acting sequences downstream to the (A/G)TAAG sequence) then dictate the differences in late and very late transcription.

Therefore, the major determinant of polyhedrin gene transcription is located at the transcription start point, and additional determinants are found between the RNA start point and the translational initiation codon.

Sequence Conservation of the Polyhedrin Promoter

The *polh* promoter sequence is relatively well conserved between otherwise distantly related baculoviruses (Rohrmann 1986). Comparison of a number of polyhedrin genes from different baculoviruses has highlighted the presence of a 12 nucleotide consensus sequence spanning the mRNA start site-(A/T)ATAAGNA(T/A/C)T(T/A)T (the transcriptionally important TAAGTATT motif of the AcNPV *polh* promoter is part of this consensus sequence; the (A/G)TAAG motif is present at the transcription start point of all late and very late promoters). This 12 nucleotide consensus sequence is also present in the very late p10 genes which have been analyzed. Another sub-group of baculoviruses (granulosis viruses) also have the same consensus sequence upstream of the granulin gene (which encodes an occlusion body protein of similar function to polyhedrin).

The promoters of the AcNPV *polh* gene and the other very late gene p10, though sharing a similar organization, display only limited sequence homology (Roelvink et al. 1992). The *polh* promoter is composed of a 50 nucleotide 5′ non-coding leader sequence and a short sequence of ~20 nucleotides upstream from the transcription start point. The AcNPV p10 promoter is composed of a 70 nucleotide 5′ non-coding leader sequence and an additional 30 nucleotides upstream from the transcription start point. The 5′ leaders of both promoters are extremely AT-rich (80%). A consensus nucleotide

sequence (AATAAGTAT) is present which includes the transcriptionally important (A/G)TAAG motif present in all late and very late promoters. Interestingly, the p10 gene has a transcriptional pattern distinct from the other very late *polh* gene —the p10 gene is active at least 4 hr earlier than the *polh* (Reoelvink et al. 1992). the promoter sequences responsible for this difference in transcriptional activity remain to be determined.

Unusual Structure of the Promoter

Transcription of the *polh* promoter may result from the virus-induced RNA polymerase recognizing the transcriptionally important TAAGTATT motif. The short promoter motif at the transcription initiation site is unusual in structure when compared to typical promoters recognized by nuclear eukaryotic RNA polymerases and bacterial RNA polymerases. Both bacterial and nuclear eukaryotic promoters usually consist of multiple non-contiguous blocks of sequence information present upstream or downstream to the transcription start point. In contrast, the motif present at the transcription start point of the *polh* promoter appears to be more similar to that of promoters recognized by RNA polymerases specific to yeast mitochondrial DNA and certain bacteriophages (T7 and T3), (Masters et al. 1987). These promoters also consist of short sequences located at the transcription start point. Yeast mitochondrial RNA polymerase, for example, recognizes and initiates at the sequence TATAAGTATT, which is remarkably, although perhaps coincidentally, similar to the polyhedrin initiation sequence. This has led Yang et al. (1991) to propose that, based on the promoters they recognize, the virus-induced RNA polymerase and mitochondrial RNA polymerase may be related.

Also, all the additional determinants of *polh* promoter activity are present in the region specifying the untranslated mRNA leader (Ooi et al. 1989) another unusual structural feature of this promoter.

A Novel Polymerase Recognizes the Polyhedrin Promoter

The polyhedrin promoter, like all late and very late promoters, is recognized by a novel, α-amanitin-resistant, baculovirus-induced RNA polymerase. Fuchs et al. (1983) monitored AcNPV-specific RNA synthesis in isolated nuclei of *Spodoptera frugiperda* cells in culture at different times post infection. They showed that early viral RNA synthesis was sensitive to 5 μg of α-amanitin per ml. During the course of infection this sensitivity decreased, and at 24hpi RNA synthesis was completely resistant to α-amanitin. Their studies revealed that the α-amanitin-resistant transcription begins just after 6hpi, simultaneous with the beginning of late phase of transcription. This novel polymerase was isolated at 24 hpi and shown to be a new, chromatographically and immunologically distinct form whose kinetics and response to divalent cations differed from those of the host RNA polymerases (Fuchs et al. 1983, Yang et al. 1991).

Fuchs et al. proposed that early in infection, the viral genes are transcribed by host RNA polymerase II. One or more early genes code for a virus-specific α-amanitin-resistant RNA polymerase or factors that modify one of the host polymerases. The virus-induced or virus-modified polymerase then plays a major role in late and very late transcription including *polh* transcription.

lef Genes are Essential for Promoter Functioning

Passarelli and Miller (1993a) developed a method to identify baculovirus genes

required for late and very late gene expression. Their method was based on the substraction of clones from an AcNPV genomic library which was able to transactivate promoters of reporter plasmids in transient expression assays. Using this method they were able to identify and sequence five early genes, designated *lef*-1, *lef*-2, *lef*-3, *lef*-4, and *lef*-5 (for late expression factor) which are essential but not sufficient for late and very late gene expression (Li et al. 1993, Passarelli & Miller 1993a, 1993b, 1993c). These *lef* genes are dispersed throughout the AcNPV genome such that *lef*-1, *lef*-2, *lef*-3, *lef*-4 and *lef*-5 are located on 7.35-8.65, 2.2-2.6, 43.4-45.2, 57.6-58.8, 64.0-65.4 map units, respectively. However, the authors have not been able to determine whether the *lef* gene products act at the level of replication, transcription, or translation. In fact, experiments carried out by Kool et al. (1994) suggest that *lef*-1 and *lef*-2 may be directly involved in viral DNA replication. Since late and very late gene expression is dependent on viral DNA replication (Rice & Miller 1986), *lef*-1 and *lef*-2 may, as a consequence, be indirectly involved in late and very late gene expression. It is also possible that the lef-genes affect the expression of other early genes which in turn are required for late gene expression.

An Unusual 30-kDa Protein Binds to Transcriptionally Important Motifs of the Polyhedrin Promoter

Gel retardation assays carried out with the *polh* promoter, using nuclear protein extracts from uninfected and very late infected (51 hpi) Sf21 cells, indicated that a host factor (the polyhedrin promoter-binding Protein or PPBP) binds specifically to the polyhedrin promoter (Burma et al. 1994). The binding site of the promoter was fine mapped to a hexanucleotide sequence (AATAAA), just upstream to the tsp which alongwith the transcriptionally important octanucleotide (TAAGTATT) was found to be necessary for PPBP-binding. PPBP could bind over a very wide salt range, (200-2000mM NaCl), at temperatures as high as 65°C, and even in the absence of non-specific DNA such as poly[d(I − C)] clearly demonstrating that it has a very high binding affinity. The apparent dissociation constant (K_d) for the specific interaction of the binding domain and PPBP was estimated to be 3.7(±0.5) pM. By UV-crosslinking and Southwestern analysis, the molecular mass of PPBP was estimated to be about 30 kDa. Phosphatase treatment completely abolished DNA binding in case of both uninfected and infected extracts. Although examples of inhibition of DNA binding by phosphorylation are quite abundant, examples of transcription factors where phosphorylation is critical for DNA binding are rather limited, making PPBP unusual in yet another respect.

PPBP has been affinity purified from nuclear extracts from uninfected Sf21 cells and experiments are underway to understand the mechanism of regulation of transcription by PPBP.

A Putative Negative Regulator Binds to the Polyhedrin Promoter

Recently, Etkin et al. (1994) have identified a 200-kDa host factor from *Spodoptera frugiperda* cells that binds specifically to the −72 to −86 region of the *polh* promoter of AcNPV. This binding activity was found in uninfected cells and also in cells during early stages of viral infection, but decreased by 18-24 hpi (concomitant with hyper-expression of the polyhedrin gene). The authors proposed that the host-encoded DNA-binding protein may act as a negative regulator and the virus may utilize this factor to control the differential expression of late *versus* very late genes. Binding of this factor

to the polyhedrin promoter could inhibit transcription by the virus induced RNA polymerase. Degradation of this factor by 18-24hpi, therefore, would have a stimulatory effect on the polyhedrin promoter. These results, however, do not imply that this is the only mechanism governing very late expression of the polyhedrin gene.

Upstream Sequences Modulate Polyhedrin Promoter-driven Transcription

We have analyzed DNA sequences, located upto 4 Kb upstream of the *polh* promoter, *vis-a-vis* their effect on a reporter gene using a transient expression system. Deletion of two regions, corresponding to 0 to 1.1 and 1.5 to 3.12 map units, reduces expression of the luciferase reporter. The homologous region *hr*1 sequence, present 3.7 kb upstream of the polyhedrin promoter in the viral genome, caused several-fold stimulation of reporter gene expression in an enhancer-like manner. Based on the effect of neighbouring sequences, we also redefined the sequence elements essential for basal activity of the polyhedrin promoter and proposed that the essential promoter elements (−69 to −1) cannot alone account for transcription from this promoter. Viral sequences further upstream of the promoter and neighbouring/distal elements, which had so far been largely ignored, exert a major influence on transcription and may account for the hyperactivation of this promoter very late in the infection cycle.

Conclusion

The primary determinant of polyhedrin promoter function is the 8 bp TAAGTATT sequence at the transcriptional start point which is essential for transcription initiation. Additional promoter determinant(s) are located in the untranslated leader region between the TAAG sequence and the initiating ATG codon of the polyhedrin ORF. Thus, the polyhedrin promoter with all the essential *cis*-acting elements is defined as a 69 bp region extending upstream from the ATG. Two hexanucleotide sequence motifs (AATAAA) present with −57 to −14 which, along with neighbouring sequences, are the targets for binding of a cellular 30-kDa polyhedrin promoter-binding protein (PPBP). PPBP has been affinity purified and appeared to be an unusual DNA-binding protein with respect to its stability, binding affinity and specificity. PPBP is phosphorylated and may have a regulatory function because de-phosphorylation abolished DNA-binding activity. Thus far, except for this cellular PPBP factor no other host or viral protein factor(s) that may be directly involved in transcription from the polyhedrin promoter has been identified. The multiplicity of regulatory circuits and the cumulative action of such controls, perhaps, reflect the desperate measure(s) which AcNPV has to adopt to overexpress the polyhedrin gene which it has to undertake for its own survival in the environment. The requirement of upstream distal and neighbouring elements for optimal expression, the involvement of a host transcription factor (PPBP), and virus-specific factors all contribute to the unusual regulation of the polyhedrin gene promoter.

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