The bountiful and baffling baculovirus: The story of polyhedrin transcription

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Baculoviruses are a unique group of eukaryotic viruses that parasitize insects. The prototype member of the family *Baculoviridae* is *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV). Global interest in baculovirus biology stems from two important uses of baculoviruses – as biopesticides and as a highly favoured eukaryotic expression system for the large-scale production of recombinant proteins in the laboratory. Of late, baculoviruses have invited renewed interest by virtue of their potential use as a delivery system in gene therapy. Although the baculovirus expression vector system (BEVS) is extensively used worldwide, the transcriptional regulation of the hyperactive promoters used to drive foreign gene expression still remains shrouded in mystery. It is clear, however, that this regulation involves an intricate interplay of both host and viral factors. This review provides an overview of what we do know about the mechanisms of transcription of baculoviral genes, with special emphasis on the polyhedrin promoter, the workhorse promoter of the BEVS, and the insect cell host factors involved in enhancing transcription from it.

Baculoviruses: Versatile and effective biopesticides

The natural hosts of baculoviruses are insects belonging mainly to the classes Lepidoptera (butterflies and moths), Hymenoptera (sawflies) and Coleoptera (beetles). Many of these insects are plant pathogens, infecting agriculturally important crops and forest trees. Although chemical pesticides continue to be used to tackle this problem, farmers and agricultural scientists have of late recognized the importance of developing a safer and more eco-friendly alternative to such harsh chemical insecticides. Baculoviruses present the perfect biological solution to curb insect pest populations while simultaneously respecting the environmental balance. The viruses can be sprayed as a powder over the crops, whereby they are ingested by the feeding insect larvae, multiply in the host and ultimately kill the organism, releasing fresh virus particles into the environment to start the cycle all over again. There are several advantages of using baculoviruses as insecticides – they can be specifically targeted to certain pests, are self-propagating, safe for human handling and do not pollute the environment, thus preventing health hazards.

However, for a variety of reasons, it has proved more difficult than expected to develop effective baculovirus insecticide formulations. The reason for this is mainly the virus's low persistence in the environment, especially when recombinant baculoviruses are used. Another major drawback is the slow speed of killing in contrast to chemical insecticides which have a much more rapid knockdown effect. The widespread use of baculoviruses as pesticides is further hindered by their narrow host range, instability of insecticide formulations, and problems in registration and patentability. However, despite these limitations, the enormous ecological advantages of this approach makes the attempt to develop new-age and more efficient baculovirus biopesticides on a global scale well worth the effort[1].

The baculovirus expression vector system: A biofactory *par excellence* for the production of recombinant proteins

The second – and far more important – use of baculoviruses is as a vehicle for large-scale protein production. Baculoviruses have been very successfully used for the past couple of decades, for the expression of high levels of recombinant proteins[2,3]. Hundreds of proteins have been expressed to date by constructing recombinant baculoviruses. The heterologous gene is expressed usually under the control of the hypertranscribed *polyhedrin* (*polh*) or *p10* gene promoters that are turned on very late following viral infection – after 48 h or so.

The polyhedrin protein forms the crystalline matrix of viral polyhedral bodies (also called polyhedra), whereas the p10 protein forms large arrays of fibrous material,

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primarily in the nucleus but sometimes in the cytoplasm as well. Polyhedra formation is crucial for viral infection of insects in the wild, since they shield the delicate virions from harsh environmental stresses. Further, polyhedra act as a useful carrier of the virus particles; the crystalline polyhedrin matrix is solubilized in the mid-gut of the insect, thus releasing the virus particles which infect the mid-gut cells. Polyhedrin is thus an extremely important protein for virus survival and propagation in its native environment. However, under laboratory conditions, where growth parameters are tailored to suit the virus, the polyhedrin matrix is no longer a prerequisite for virus survival. Thus, the *polh* gene can be replaced with a heterologous gene of choice, which would then be efficiently expressed from the strong *polh* promoter. The same logic holds good for the *p10* gene. The hypertranscribed *polh* and *p10* promoters are thus the workhorse promoters of the baculovirus expression vector system (BEVS).

The BEVS owes its popularity to many more qualities other than just the unusual strength of the *polh* and *p10* promoters. Some of these are described briefly below:

Eukaryotic environment for protein production

The BEVS provides the necessary higher eukaryotic environment essential for the proper folding, post-translational modification, disulphide bond formation and other modifications required for the functional activity of many eukaryotic proteins. Post-translational modifications that have been reported to occur in the insect BEVS include signal cleavage, proteolytic cleavage, N-glycosylation, O-glycosylation, acylation, amidation, phosphorylation, prenylation and carboxymethylation[4]. All these modifications occur at sites identical to those in the wild type proteins, reinforcing the usefulness of the BEVS as one of the most favoured systems for expressing functionally active recombinant proteins.

Extremely high levels of expression

As mentioned earlier, the *p10* and *polh* promoters are the most commonly used promoters to drive the expression of foreign genes. Being unusually strong promoters, they hypertranscribe the gene(s) put under their control to the extent that the recombinant protein can account for about 25–50% of the total cellular protein. The *polh* promoter is the stronger of the two, and induces higher expression levels, but investigators have reported protein yields of up to a gram of recombinant protein her litre of insect cell culture, i.e. about 10^9 cells, using either promoter. Average protein yields lie in the range of 10–100 mg of protein per 10^9 cells. In terms of protein yield alone, the BEVS has been observed to consistently outperform other expression systems. The use of homologous enhancer-like sequences has been demonstrated to further enhance foreign gene expression levels several fold[5]. In addition, live caterpillars have also been used as a host for high level expression, to further improve on the economics of expression[6].

However, the expression kinetics differ from protein to protein and the promoter used[7], with the time of expression playing a critical role in proper post-translational modification and secretion[8]. Expression levels are also known to vary with the cell line used[9]. Another important parameter is the codon usage pattern of the recombinant gene: heterologous genes which use non-optimal codons of the insect host are observed to be poorly expressed[10,11]. Finally, the translation initiation context – as defined by the Ranjan–Hasnain consensus sequence[11] – was also found to play a major role in regulating protein expression levels. Recently, a recombinant baculovirus carrying different B and T cell epitopes from nine stage-specific antigens of *Plasmodium falciparum*, has been used to express, to very high levels, a recombinant multi-antigenic protein – a strong putative vaccine candidate for malaria[12,13].

Capacity for large inserts and simultaneous expression

The baculovirus nucleocapsid is predicted to be capable of accommodating inserts as large as 100 kb. Although this has not been practically tested (the largest insertion till date being only about 15 kb)[4], no investigator has been hampered by the size of the heterologous gene(s) used for insertion. Further, a number of transfer vectors have been genetically engineered to simultaneously express multiple genes under the control of different viral promoters both in cell culture[14–16] and in *Spodoptera* larvae[17].

Baculovirus-mediated gene transfer into mammalian cells

The BEVS has so far used only insect cells as a host for the expression of heterologous genes carried by recombinant baculoviruses. Interestingly, recent reports have demonstrated that they could also be used as gene delivery systems in mammalian cells. Although baculoviruses infect over 30 species of Lepidopteran insects, they are incapable of replicating in other insects or in any of over 35 mammalian cell lines studied[18,19]. However, the virus does enter mammalian cells and the viral DNA is capable of reaching the nucleus. Experimental studies have shown that when an exogenous promoter, such as that derived from Rous sarcoma virus or cytomegalovirus, is inserted into the baculovirus genome,

the modified virus becomes capable of gene expression in non-Lepidopteran cell lines, including various mammalian cells[20,21]. Boyce and coworkers[20] showed that reporter gene expression from a recombinant baculovirus was significantly higher in the human hepatocellular carcinoma cell line, HepG2, than in cell lines derived from other tissues like monkey kidney, human kidney, cervix and lung, B-cell, T-cell, rat adrenal, and mouse embryo fibroblast and muscle. Thus, new generation recombinant baculoviruses could well evolve into a gene delivery system of the future[22].

Safety and simplicity

Baculoviruses are relatively simple to use. Constructing recombinant viruses is much faster and easier than developing stable recombinant high-expressing cell lines, and the host insect cells can be grown at 27°C either as adherent or suspension cultures. Cells can be grown in a BOD incubator since CO_2 is not required for growth. Scale up has also been perfected with time, thus making it easy to produce large amounts of host insect cells in fermenters for subsequent viral infection and expression of recombinant proteins. Further, since baculoviruses have no non-arthropod hosts *in vivo*, they are harmless to humans and can be safely handled by investigators with no special precautions. However, a recent report by Gronowski *et al.*[23], have shown that *Ac*NPV is capable of provoking an anti-viral response in murine and human cell lines by inducing interferons. Although the possibility of baculoviruses infecting humans *in vivo* is remote, these findings nevertheless justify the use of greater precautionary measures in handling baculoviruses than in the past.

Gene expression in baculoviruses

During an NPV infection, more than a hundred viral genes are expressed in a cascade that can be broadly divided into three stages – early, late and very late. Each stage is characterized by the expression of a unique set of genes in a well-regulated cascade, with the products of one group of genes required for the expression of the next set[24]. By definition, early genes are expressed prior to viral DNA replication. Most, if not all, immediate-early genes encode transcriptional regulatory proteins[25–29]. DNA replication activates the viral template in a manner not yet defined and enables the late and very late classes of genes to be expressed, which encode proteins essential for virion assembly and viral occlusion formation. Although one of the most intriguing aspects of baculovirus biology concerns the control of the viral transcription cascade, we are still a long way from deciphering the precise mechanisms involved and the host and viral factors which play a role in this finely-orchestrated process.

Early gene expression

Early viral gene expression spans the time period from 0–6 h post-infection (hpi) and results in the transcription of genes encoding proteins required for viral DNA replication and late gene transcription. Most baculovirus early genes have a TATA box located 25 to 30 bp upstream of a conserved mRNA transcription initiation site that consists of the sequence CAGT[30]. The CAGT element, which is the first true initiator element to be discovered in baculoviruses, has been shown to be critical for the transcription of early genes. Substitution of the CAGT sequence resulted in a reduction of both reporter activity and *in vitro* transcripts, although transcripts initiated accurately[31]. However, not all early genes have a CAGT and/or TATA element. A notable example is the *dnapol* gene in both *Bm*NPV and *Ac*NPV, which is observed to initiate transcription from a GC rich region, with no canonical TATA box or CAGT motif present[31]. Early gene expression is dependent on a α-amanitin sensitive, tagetitoxin-insensitive host RNA polymerase II (ref. 32) and early gene promoters resemble typical eukaryotic class II promoters.

Four *Ac*NPV early genes, *ie-O*, *ie-1*, *ie-2* (or *ie-N*), and *pe-38*, have been shown to be important for transactivating early baculovirus promoters in transient expression assays. IE-0, IE-2, and PE-38 mRNAs are expressed only during the early phase of infection[25,33,34]. In contrast, IE-1 RNA is expressed during both the early and late phases of infection[28,33].

IE-1, a 582-amino acid long multifunctional transcriptional regulatory phosphoprotein, has been shown to transactivate a number of delayed-early genes including *39K*, *ie-2* and *p35* (refs 26–28) and at least one late gene, *39K* (ref. 29). The stimulatory effect of IE-1 is greatest when the target promoter is *cis*-linked to *Ac*NPV homologous repeat regions (*hrs*). Hr regions, which are present at nine dispersed locations in the *Ac*NPV genome, have been shown to act as enhancers of transcription[25,28,35–37] and also in certain cases as origins of viral replication[38–40]. However, competitive PCR methods used to map the activation profiles of *Ac*NPV *oris*, have demonstrated that the *ie-1* gene promoter also acts as an *ori*, and is activated in a temporal fashion[41]. IE1 has been shown to also negatively regulate certain promoter regions, e.g. the promoters of the *Ac*NPV *ie-0* and *ie-2* genes, which themselves are regulatory genes. Results indicate that IE1 brings about this downregulation of transcription by binding directly or as part of a complex to IE-1 binding motifs (5′-ACBYGTAA-3′) near the mRNA start site[42]. In addi-

tion, it has been shown that *ie-1* can activate the *he-65* gene promoter in insect *Trichoplusia ni* (*T. ni*) 368 and mammalian BHK21 cells[43], demonstrating that the activity of this multifaceted protein is not confined to a narrow host range, but probably involves a generalized mechanism conserved across various species.

Several other baculovirus early genes also have important regulatory functions. IE-0 is a protein identical to IE-1 except that it has an additional 54 amino acids at the N terminus as a result of a splicing event[33]. IE-0 mediated activation has been shown to require an *hr* enhancer[44] unlike IE-1, which can regulate transcription in the presence or absence of a *cis*-linked hr sequence. A further difference between IE-0 and IE-1 is that the former does not appear to downregulate the *ie-0* promoter[44].

ie-2, another early regulatory gene, both augments *ie-1* transactivation[25] and brings about transcription activation independent of *ie-1* (refs 26, 45). IE-2 activates the *ie-1* promoter approximately 2.5-fold in transient expression assays[45,46] and has been demonstrated to block cell cycle progression in a variety of cell lines, including *Sf*21 and *T. ni*[46].

The *pe-38* gene has been shown to transactivate the *p143* gene promoter[36]. Other *Ac*NPV genes such as *me-53* and *cg-30* are also thought to have some role in activating gene expression, based on their sequence similarity with transcriptional regulators from other systems[47,48].

The *p35* gene, an inhibitor of members of the ICE family of cysteine proteases and a major determinant of virus host range, is another essential early gene. p35 is required to suppress the apoptotic response of *S. frugiperda* cells to *Ac*NPV infection by acting as a suicide inhibitor of caspases[49,50], as well as an antioxidant[51,52], both in *Sf*21 and *Sf*9 cells and *S. frugiperda* larvae[49]. The important role played by the *p35* gene suggests that effective inhibition of apoptosis is required for both efficient viral DNA replication and gene expression[53,54].

The early *p143* gene is another important player in viral replication[53,55], transcription, shutdown of host protein synthesis and viral host range determination[54–56]. p143 has helicase-like motifs, a nuclear localization signal and a leucine zipper motif, is synthesized in virus-infected cells prior to the initiation of viral DNA replication and has been shown to bind non-specifically to DNA[57]. *In vivo* complementation assays[58] revealed that some of the putative helicase motifs are not essential for p143 function; however, mutations within an ATP-binding motif, a potential helix-turn-helix region, and certain large amino acid deletions inactivated protein function. Recent reports have confirmed that p143 is indeed a DNA helicase with ATPase activity[59,60]. LEF-3, a single-stranded DNA-binding protein, has been shown to interact with p143 and help in localizing p143 to the nucleus[61]. p143 is also a crucial determinant of viral host range; interspecific replacement of a short sequence in the *Ac*NPV *p143* gene renders the virus capable of infecting *Bombyx mori* larvae[59]. It has been demonstrated that two key mutations in the *Ac*NPV p143 host specificity domain is enough to render the virus replication competent in *Bm5* cells, and kill *B. mori* larvae.

Finally, *orf121*, another baculovirus early gene has been observed to stimulate expression from the late *39K* gene promoter, an activity which is dependent on *ie-1* as well[62]. It was later discovered that *orf121* enhanced *ie-1* expression which in turn was responsible for upregulating expression of the *39K* gene[62].

Late and very late gene expression

The transition between early and late viral gene expression is the most distinctive regulatory event in the baculovirus transcription/infection cascade. Whereas early gene expression is dependent on an α-amanitin sensitive RNA polymerase present in uninfected cells, late and very late gene transcription involves a novel α-amanitin and tagetitoxin resistant, virally encoded RNA polymerase[32,63–66].

The baculovirus late and very late gene promoters resemble mitochondrial and bacteriophage T7 late promoters in that a short conserved sequence serves both as a promoter and an initiator element. The most conserved sequence element of *Ac*NPV late and very late promoters is the transcription initiator (A/T/G)TAAG[30]. The strength of expression from the promoters is critically dependent on the context of the TAAG sequence, with the 18 bp region encompassing the TAAG having been shown to be the minimal promoter determinant for basal transcription from the late *vp39* promoter[67]. However, essential promoter determinants for the very late *polh* and *p10* genes include not only the 12-bp initiator AATAAGTATTTT but also a downstream A + T rich region corresponding to the 5′ untranslated leader sequence of their mRNAs[68,69]. This untranslated sequence is responsible for the 'burst' in transcription observed during very late gene expression and is thus termed the 'burst' sequence. It is a defining element for very late gene promoters, being absent in late promoters. Mutations within the burst sequence reduce expression during the very late phase of infection by 10 to 20-fold and lower both the steady-state levels of *polh* mRNA and the rate of transcription initiation from the *polh* promoter[68]. In contrast, mutations in sequences upstream of the *polh* promoter TAAG motif have relatively milder effects on *polh* gene expression[69]. Progressive deletions of the *p10* promoter also suggest the presence of a burst sequence that is essential for strong expression during the very late phase[69].

The *Ac*NPV very late factor 1 (*vlf-1*) gene has been shown recently to play an important role in very late gene expression from the *polh* and *p10* gene promoters. *vlf-1*, a late gene, was originally identified by characterization of an occlusion-defective mutant virus, *ts*B837, which produced extremely low levels of *polh* and *p10* transcripts during the very late phase of infection. In transient expression assays, *vlf-1* was shown to stimulate expression from very late promoters but not from late promoters[70], making it probably the only well characterized viral factor known to date that regulates only very late but not late gene expression. Interestingly, recombinant baculoviruses with altered *vlf-1* expression revealed that the time of *vlf-1* expression and/or the concentration of VLF-1 in the cell was critical for switching on the *polh* and *p10* genes[71,72]. Recent studies have revealed that the VLF-1 protein binds directly to the untranslated regions of the polh and p10 promoters and is closely correlated with their transactivation[73]. Thus, VLF-1 seems to be a crucial player in the regulation of baculovirus very late gene expression.

Until recently, eighteen *Ac*NPV genes, called *lef*s (late expression factors), were identified[74–77] which are necessary to support transient expression of a reporter gene under the control of the late *vp39* promoter. The same set of *lefs* has also been shown to be involved in transient expression of the late basic 6.9-kDa protein gene[70] and the very late *polh*[68] and *p10* genes[70]. All these genes have been shown to affect steady state levels to reporter gene transcripts, implying that their effect is mainly at the transcriptional and not at the translational level[54].

Nine of the 18 genes (*ie-1*, *ie-2*, *lef-1*, *lef-2*, *lef-3*, *lef7*, *p143* (also called *dnahel*), *dnapol*, and *p35*) are necesary and sufficient for supporting replication of a plasmid containing a viral origin of replication[54]. Hence, they may act indirectly, by supporting viral DNA replication post-infection, which is also essential for late and very late gene expression.

The *ie-1* and *ie-2* genes, as explained above, are important immediate early genes which regulate the expression of their own genes and other immediate – and delayed early genes in the viral cascade. *p143* (*dnahel*) and *dnapol* encode polypeptides with sequence similarity to DNA helicases and polymerases, respectively, suggestive of their role in viral DNA replication. The viral late expression factor 3 (*lef-3*) gene product has been shown to have single-stranded DNA binding activity[78]. LEF-3 forms a homotrimer in solution[79] and helps to localize the P143 DNA helicase to the nucleus[61]. The *Bm*NPV LEF-3 protein has been demonstrated to have a helix-destabilizing activity, which may act in concert with P143 to facilitate strand separation during DNA replication. The involvement of *p35*, a known inhibitor of apoptosis, suggests that the apoptotic pathway needs to be blocked in order for DNA replication and subsequent transcription to occur.

The *lef-1*, *lef-2* and *lef-7* genes have been shown to be essential for DNA replication. The LEF-1 protein has primase-like motifs, which when mutated abrogates the ability of LEF-1 to support transient DNA replication[80]. Furthermore, two-hybrid screens have demonstrated, although the exact mechanism is unclear, that LEF-1 and LEF-2 interact with each other, and probably function synergistically in the replication process[80]. The *lef-2* gene also plays an important role in replication[56]. Merrington and coworkers[81] identified a mutant virus, VLD1 which was defective in late and very late gene expression which was subsequently found to be the result of a point mutation in the *lef-2* gene. Interestingly, the virus was not defective in DNA replication, suggesting that *lef-2* may play a dual role, both in DNA replication and very late gene expression, with different domains of the protein required for different functional roles[81]. The *lef-7* gene has been demonstrated not to be absolutely essential for DNA replication, but instead have a stimulatory effect. *lef-7*, like *lef-3*, is dependent on the multifunctional trans-regulatory gene, *ie-1*, for its activity in transient expression assays[75].

The remaining 9 *lefs*, i.e. *lef-4*, *lef-5*, *lef-6*, *lef8*, *lef-9*, *lef-10*, *lef-11*, *39K* and *p47* are directly involved in regulating late and very late gene expression, and not simply as a consequence of supporting DNA replication.

The *39K* gene encodes a phosphoprotein, pp31, which associates with the virogenic stroma, a virus-induced nuclear structure which appears to be the site for nucleocapsid assembly[82]. *39K*, like several other genes, is also regulated by the transactivator IE-1. ORF121 and IE-2 also upregulate expression of 39K, although this was found to be by virtue of their enhancement of IE-1 expression[62]. P35 also enhances expression of 39K by a mechanism which is thought to be different from that adopted by ORF 121, IE2 and IE-1 (ref. 62).

Recently, the virus-specific RNA polymerase was purified[63] and was apparently found to be composed of equimolar subunits of 4 lefs: LEF-4, LEF-8, LEF-9, and p47. LEF-8 and LEF-9 were earlier described as having some, though not extensive, sequence similarity with other DNA-dependent RNA polymerases[74,77]. This polymerase has been suggested to be the simplest DNA-directed RNA polymerase reported till date from any eukaryotic source. The polymerase supported transcription from late and very late promoters but was not active on early promoters. Interestingly, both late and very late promoters were transcribed with equal efficienty, highlighting the fact that the polymerase lacked the factors to bring about temporal expression of the late and very late genes in the sequential order required during the viral cascade of infection. Significantly, this complex of 4 equimolar subunits has not been shown to

function in *in vitro* reconstitution experiments but has been demonstrated to act only within the insect cell environment. The role of other host factor(s) in this process, *in vivo*, has thus not been excluded.

The LEF-4 protein was recently shown to have guanylyltransferase activity. It could hydrolyse the gamma-terminal phosphate of the 3′ end of RNA and also ATP and GTP to their respective dinucleotide forms[83]. These activities and the fact that LEF-4 has a KXDG motif, and homology with motifs common to viral and cellular guanylyltransferases, suggest that it may be part of a baculovirus RNA-capping complex. The triphosphatase, guanylyltransferse and methyltransferase components of the capping apparatus are organized differently in metazoan, viral and fungal systems. However, vaccinia virus capping enzyme has been shown to combine all three properties in a single multifunctional protein. LEF-4 combines the first two functions of the capping apparatus and is thus thought to be the major player in baculoviral mRNA capping[83] besides being part of the core RNA polymerase complex.

Of the remaining constituents of the viral RNA polymerase, the LEF-8 and LEF-9 proteins are hypothesized to constitute the catalytic core of the RNA polymerase since they possess amino acid sequence motifs with homology to other polymerases[63]. However this has not been proved experimentally, and the role of the p47 protein is still unknown.

No information is currently available on the functions of *lef-6*, *lef-10* and *lef-11* and the mechanism by which they are involved in late and very late gene expression. Although the exact role of *lef-5* too has not been delineated, it has been recently reported that the C-terminal end of the protein contains a novel domain which is homologous to the zinc ribbon domain of RNA polymerase elongation factor IIS (TFIIS) from a variety of taxa[84]. The same report also documents the interaction of the LEF-5 protein with itself and suggests that LEF-5 may be involved in transcript elongation.

Apart from these 18 *lefs*, a new factor, *lef* 12, has been identified of late, which is also supposed to be essential for late and very late gene transcription[85]. A set of plasmids was constructed in which each of the 18 *lef* open reading frames (ORFs) was controlled by the *Drosophila melanogaster* heat shock protein 70 (hsp70) promoter and epitope tagged. However, this set of plasmids failed to support transient late gene expression. The inability of the *p47* ORF to replace the *p47*-containing plasmid supplied in the *lef* plasmid library led to the identification of a 19th late expression factor gene (*lef-12*) located adjacent to the *p47* gene. The sequence of *lef-12* is predicted to encode a 21 kDa protein with no homology to any previously identified protein. The function of *lef-12* is yet to be elucidated.

Role of host factors in very late gene expression

All of the factors with an established role so far in viral late and very late gene expression, including the *polh* and *p10* genes, are viral proteins. However, many insect cell host factors too are critically important for this process. Our laboratory has been working on this aspect for the past several years and has identified several cellular proteins which play a crucial role in very late gene expression, using the *polh* promoter as a model[86].

Etkin and coworkers[87] had earlier identified a 200 kDa protein present in *Sf9* cells which binds to the polh promoter and was implicated in negative regulation of the promoter. However, the functional importance of this putative factor was not elucidated and there were no further reports about it thereafter.

The first host protein to be clearly identified as having an authentic role in *polh* transformation is the unusual 30-kDa transcription factor, the polh promoter binding protein (PPBP) identified in our laboratory[88,89]. This phosphoprotein binds with very high affinity and specificity to a hexanucleotide sequence motif, AATAAA, present within the minimal promoter immediately 5′ to the octanucleotide motif TAAGTATT which encompasses the transcription start point[90]. PPBP probably acts as an initiator binding protein (IBP) involved in the recruitment of the transcription machinery. PPBP specifically binds to the coding strand of the promoter[91] with increased affinity, compared to the duplex promoter, thus maintaining the promoter at the initiation point in a 'melted' state and allowing for increased rounds of transcription. Sequestering PPBP using its cognate binding motif – the polh promoter B domain – resulted in a drastic reduction in transcription

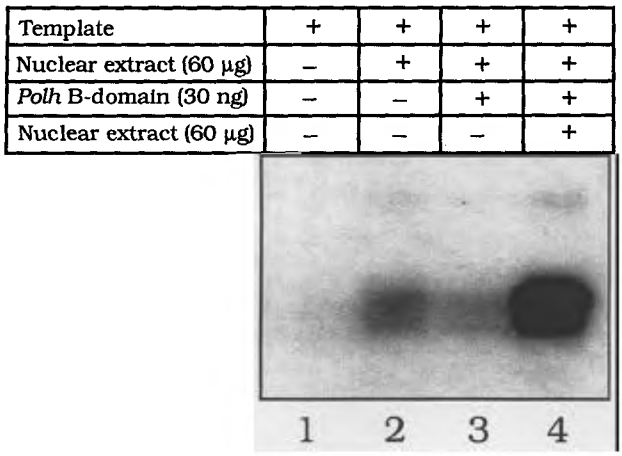

Figure 1. PPBP is required for transcription from the polh promoter. *In vitro* transcription from Ppolh was carried out using a C-free template plasmid. Transcription was reduced drastically when PPBP was titrated out using its cognate binding motif (compare lanes 3 and 2) and restored when the reaction was replenished with *Sf*9 nuclear extract containing PPBP (lane 4). Lane 1 is a control reaction carried out in the absence of template DNA.

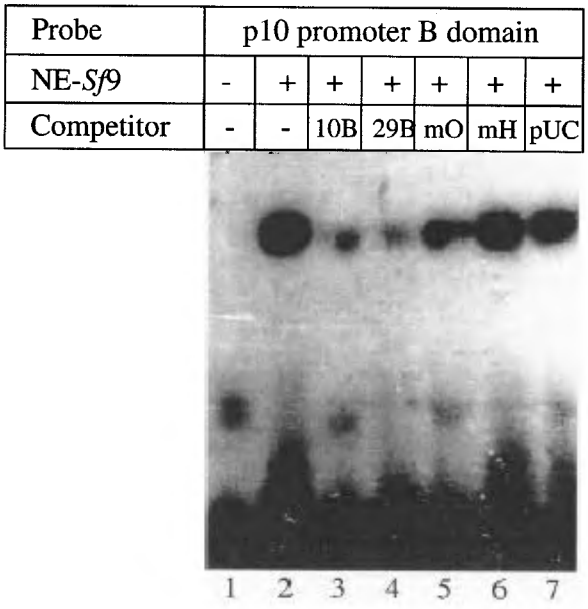

Figure 2. PPBP also binds to the *p10* gene promoter. EMSA showing a PPBP–DNA complex using the p10 B domain promoter sequence as probe. The complex (lane 2) could be specifically competed out using either the cold p10 or p29 (polh) promoter sequences (lanes 3 and 4, respectively) but not by polh promoter oligonucleotides carrying mutations in the TAAGTATT (mO) or AATAAA (mH) motifs (lanes 5 and 6, respectively). pUC18 DNA, used as a non-specific competitor, did not compete for binding either (lane 7). Lane 1 represents the free probe.

in vitro and *in vivo*[90] (Figure 1). Interestingly, PPBP has been shown to interact with the transcriptionally important AATAAA and TAAGTATT motifs of the *p10* promoter also[92] (Figure 2), suggesting that it may have a role in the regulation of gene expression from very late promoters in general (Jain, A., Ph D thesis).

The second host factor identified in our laboratory is the 38 kDa homologous region-1 (*hr1*) binding protein (hrBP), which binds at multiple sites within the *Ac*NPV *hr1* enhancer element, with high specificity and affinity[37]. There are nine homologous region sequences (*hrs*) dispersed throughout the *Ac*NPV genome, viz. *hr1*, *hr1a*, *hr2*, *hr2a*, *hr3*, *hr4a*, *hr4b*, *hr4c* and *hr5* (refs 93, 94), which act as origins of replication (*ori*s) as well as enhancers of transcription for some baculovirus early and late genes. Our laboratory has shown that the *hr1* enhancer enhances transcription from the *polh* promoter and also act as a putative *ori*, with both functions having distinct sequence requirements[40]. The 750 bp hr1 sequence element contains 5 imperfect palindromes with an *Eco*RI site at the centre of each palindrome. An intact palindrome along with the flanking sequence is the minimal requirement for the enhancer function of hr1 (Figure 3, ref. 40). This is in contrast to its replication function, where a palindrome alone was found to be both necessary and sufficient for the *ori* function of hr1 in transfected cells[40]. Hr1BP requires phosphorylation

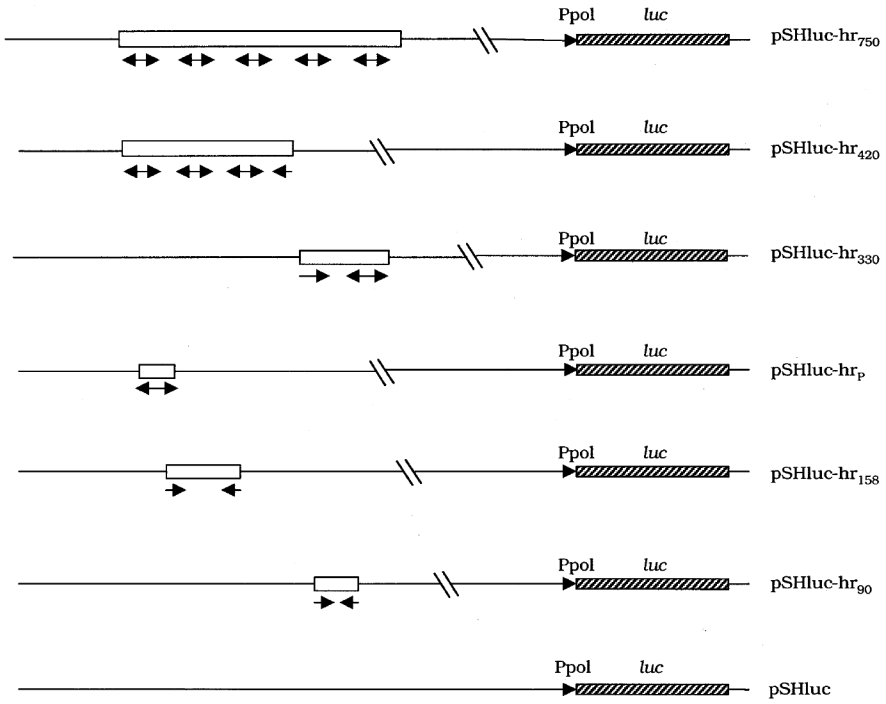

Figure 3*a*. Enhancement of luciferase expression is a function of hr1 modules. Schematic representation of plasmid constructs carrying different components of hr1 used for analysis in transient expression assays.

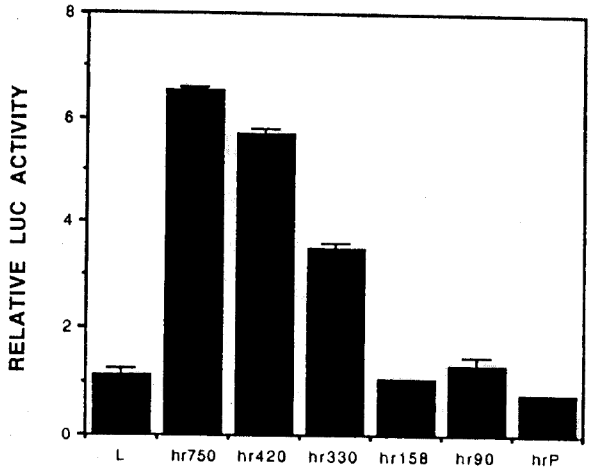

Figure 3 *b*. Enhancement of luciferase expression is a function of hr1 modules. Luciferase activity from these constructs represented as fold-enhancement over pSHluc (L). The different hr reporter constructs are indicated.

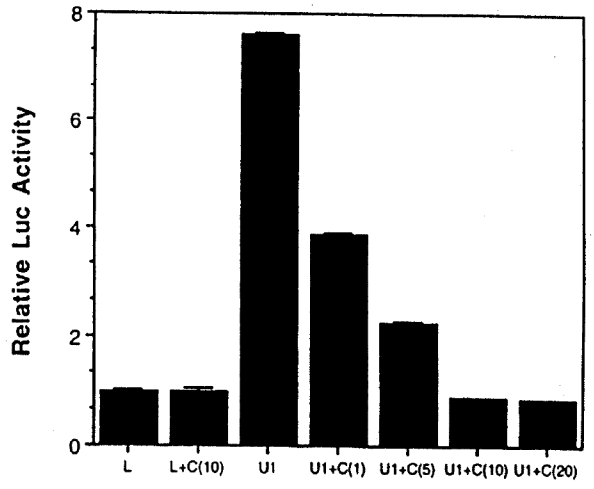

Figure 4. Hr1BP is required for the enhancer function of hr1. Luciferase expression of hr1-containing constructs (pSHluc-hrU1, U1) co-transfected with varying amounts of competitor plasmid (C, pSH-hrU1) carrying the hr1 sequence (bars 3–7). Bars, 4, 5, 6, and 7, corresponding to 1, 5, 10, and 20 µg of competitor plasmid respectively, show a drop in luciferase expression relative to bar 3, which has no competitor. Bars 1 and 2 are controls showing that there is no effect on luc expression with (lane 2) or without (lane 1) competitor in the case of a reporter plasmid which carries no hr1 sequence (L, pSHluc).

for binding and is essential for the enhancer function of *hr1*, as demonstrated by *in vivo* competition experiments[37] (Figure 4).

The host factor PPBP, which binds to the polh promoter and probably helps recruit the RNA polymerase[90], specifically recognizes the transcriptionally important AATAAA and TAAGTATT motifs within the polh promoter. Promoter vector constructs (Figure 5, ref. 95) were made, where the AATAAA was mutated to CCCCCC and the mutant promoter used to drive transcription of a downstream luciferase reporter gene. It was expected that reporter gene expression would be drastically affected, if not reduced to zero. Surprisingly, however, the drop in luciferase reporter expression (Figure 5, ref. 95) from the mutant promoter construct *vis-à-vis* the wild type promoter was not as sharp as expected. On closer analysis, it was apparent that an ~ 766 bp stretch present upstream of Ppolh could compensate for mutations within the promoter. When a control plasmid containing the mutant promoter with no upstream regions was analysed, it showed almost no luciferase expression, underscoring the importance of upstream sequences in the regulation of Ppolh. In a separate experiment, *Bal*31 deletion analyses of a 4 kb region upstream of the *polh* promoter identified two transcriptionally important regions, region I and region II (containing the 766 bp upstream sequence), spanning map units 0 to 1.5 and 2.5 to 3.12 respectively on the *Eco*RI 'I' fragment of the viral genome (Ch. Anser Azim, unpublished data). The deletion of these regions resulted in a significant reduction in *polh* promoter-driven reporter gene expression. These findings, coupled with the 'promoter-knockout' analysis results described above, promoted a more detailed dissection of region II.

The Sp family of proteins

A careful analysis of region II revealed a sequence motif, which we termed AcSp (for *Autographa californica* nuclear polyhedrosis virus Sp-like sequence), which carried GC and GT box-like motifs which are known to be bound by the Sp family of proteins (Figure 6). Keeping in mind the functional significance of the upstream sequences and the fact that Sp-family proteins have so far not been demonstrated in insect cells, it was pertinent to explore this region further to determine the importance, if any, of the AcSp sequence notif and any trans-acting factors that may bind to it. It was observed that AcSp and the consensus Sp1 sequence (cSp) specifically bound factor(s) in HeLa and *Spodoptera frugiperda* (*Sf*9) insect cell nuclear extracts to generate identical binding patterns, indicating the similar nature of the factor(s) interacting with these sequences. Recombinant plasmid constructs carrying the AcSp and cSp oligonucleotides enhanced *in vivo* expression of a polh promoter-driven luciferase gene (Figure 7). *In vivo* mopping of these factor(s) significantly reduced transcription from the polh promoter (Figure 8, ref. 95), and recombinant viruses carrying deletions in the upstream sequences containing AcSp confirmed the requirement of these factor(s) in polh promoter-driven transcription in the viral context (Figure 9, ref. 95). Our results thus document, for the first time, DNA–protein interactions involving novel members of the Sp-family of proteins in adult insect cells and their involvement in transcription from the polh promoter[95].

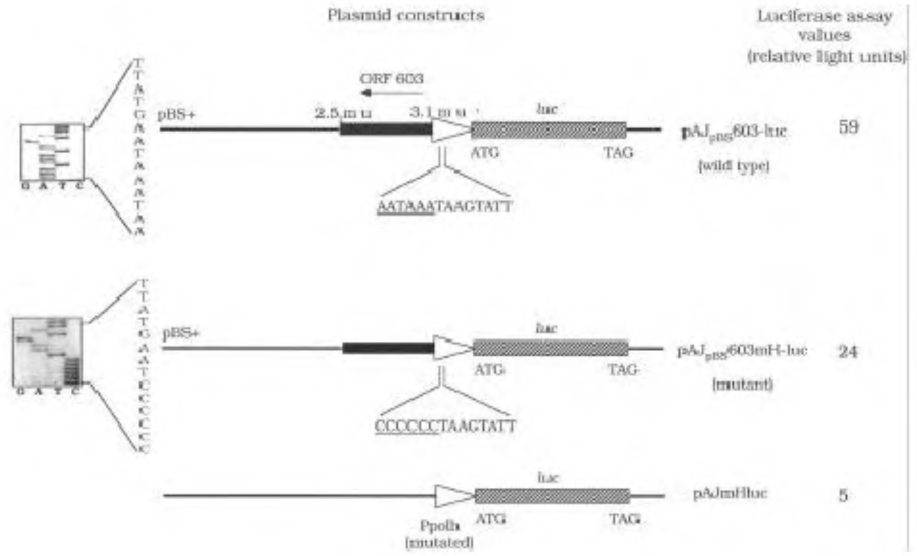

Figure 5. A ~ 766 bp region (containing the ORF603) can compensate for mutations within Ppolh. pAJpBS603-luc contains the wild type Ppolh driving expression of a luciferase reporter, with a ~ 766 bp upstream region. PAJpBS603mH-luc and pAJmHluc are the corresponding plasmids carrying a mutated promoter, with and without the 766 bp region, respectively. The corresponding luciferase values are shown alongside.

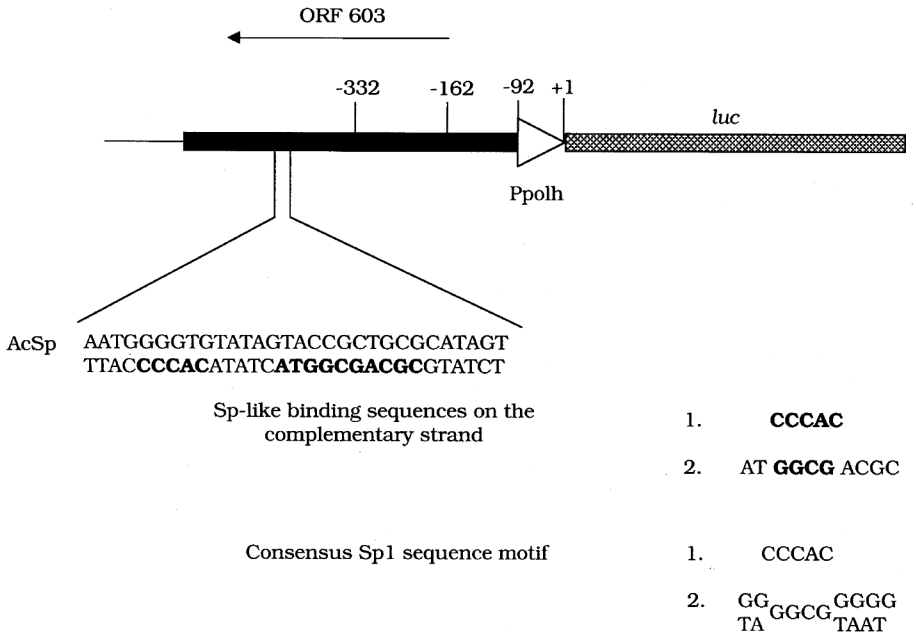

Figure 6. An Sp-like binding sequence (AcSp) is present upstream of Ppolh. The AcSp sequence bears a consensus CACCC motif and a loose GC box, depicted in boldface, on the non-coding strand.

One of the most common regulatory elements present in eukaryotic promoters, enhancers and locus control regions is the GC box (GGGCGG), or the related GT(GGGTGG)/CACCC boxes. The first major advance in our understanding of how these sequences contribute to the control of gene expression was the isolation and identification of the GC-box binding protein, Sp1. As it turned out, Sp1 is simply the first cloned and identified member of a large and still growing family of proteins which bind to similar GC/GT box sequences and share a highly conserved DNA-binding zinc finger domain[96,97]. The superfamily is referred to as the Sp or XKLF (Krüppel-like factor) family, since the zinc finger DNA binding domains of all the members share homology with those found in the *Drosophila melanogaster* regulator protein Krüppel[97]. Currently, the Sp/XKLF family comprises at least 16 different mammalian family members, and is rapidly expanding. Although some Sp-like

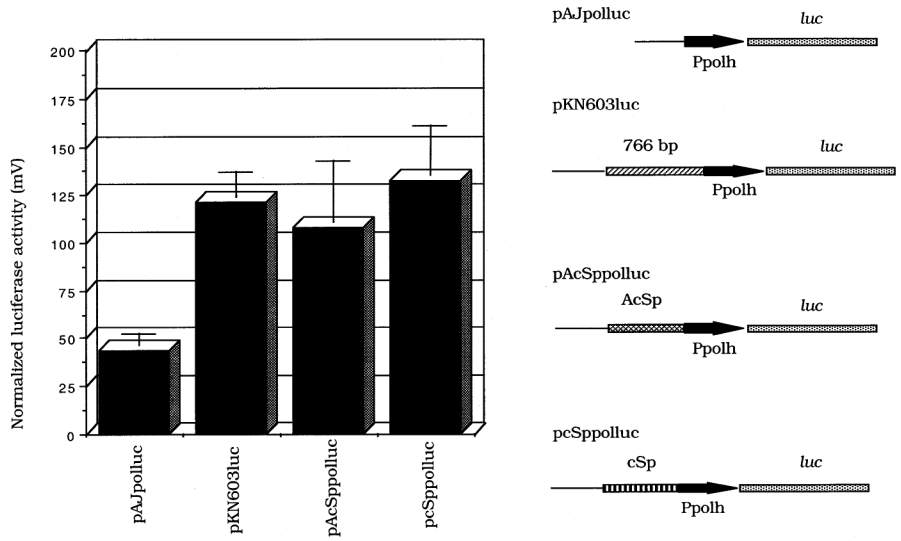

Figure 7. The AcSp and cSp sequence motifs enhance reporter gene expression when placed upstream to the polyhedrin promoter. Luciferase activity in transient expression analyses using pAcSp.pol.luc and pcSp.pol.luc, carrying the AcSp and cSp oligonucleotides respectively, upstream of the polh promoter. The relative luciferase levels of pcSp.pol.luc and pAcSp.pol.luc were compared with those of pAJpolluc (with no upstream sequences) and pKN603luc (carrying ~ 4 kb upstream sequences).

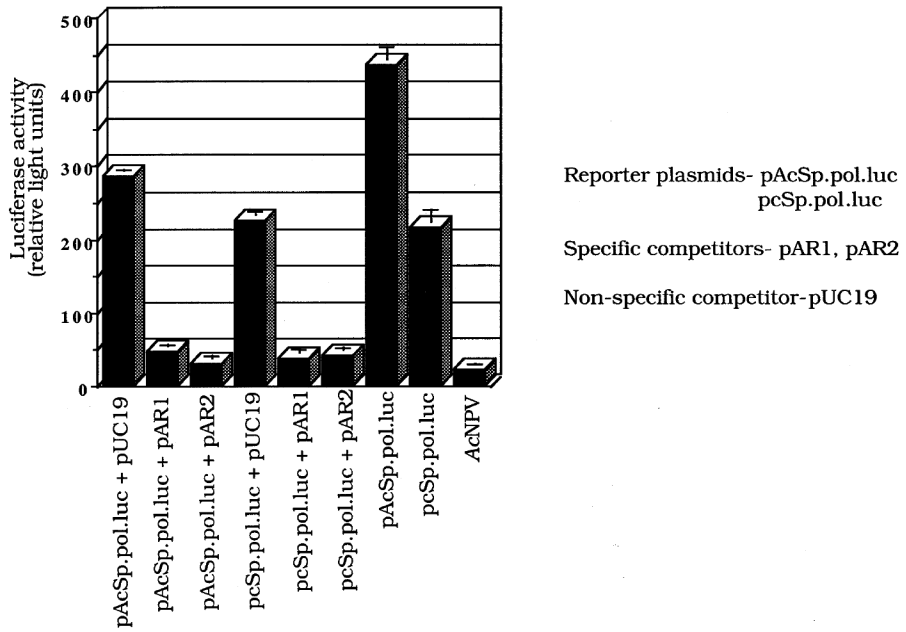

Figure 8. Mopping of the insect Sp-family protein(s) *in vivo* causes a reduction in polh promoter-driven reporter gene expression. Luciferase expression levels using 20 μg of pAcSp.pol.luc (lane 7) and pcSp.pol.luc (lane 8) plasmids after transfection into *Sf*9 cells were compared in the presence of specific or non-specific co-transfected competitor plasmids. Lanes 1 and 4 show luciferase expression using the reporter plasmids pAcSp.pol.luc and pcSp.pol.luc respectively, with pUC19 used as a non-specific competitor. Lanes 2 and 3 depict luciferase expression using pAcSp.pol.luc in the presence of competitor plasmids pAR1 or pAR2 respectively. Likewise, the competition with pAR1 or pAR2 using pcSp.pol.luc as reporter is shown in lanes 5 and 6 respectively. Lane 9 depicts *Ac*NPV infection carried out in the absence of any transfected plasmid. pAR1 and pAR2 plasmids carry the AcSp and cSp oligonucleotides respectively, cloned into pUC19.

proteins have been identified in *Drosophila* embryos only during the blastoderm stage, there are no reports of such factors being present in adult insect tissue.

Our findings show both an enhancement (in the case of an intact promoter) and a rescue of transcription (in the presence of a mutant promoter) with Ppolh upstream

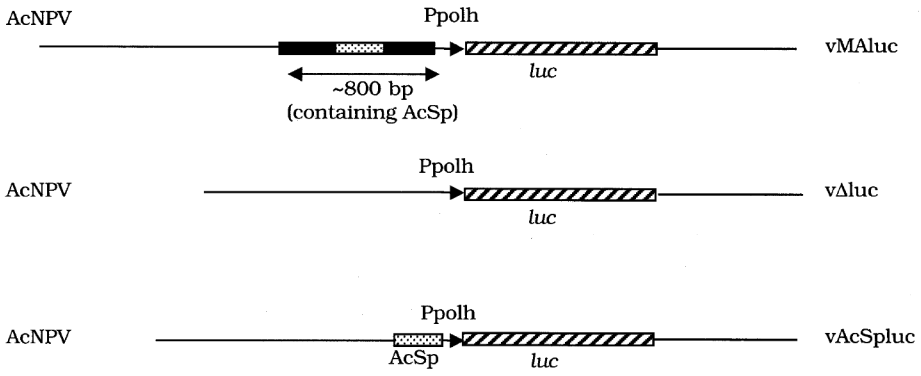

Figure 9 *a*. AcSp is required for enhancement of transcription from the polh promoter in the viral context. Schematic representation of the recombinant baculoviruses vMAluc, vΔluc and vAcSpluc carrying the polyhedrin promoter-driven luciferase gene, with varying sizes of upstream sequences.

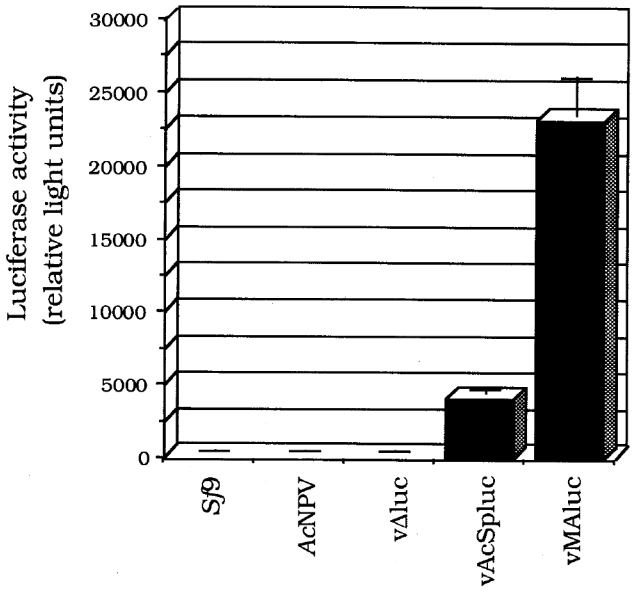

Figure 9 *b*. AcSp is required for enhancement of transcription from the polh promoter in the viral context. Luciferase levels recorded in *Sf*9 cells, or after infection with *Ac*NPV or recombinant viruses, assayed 65 hpi.

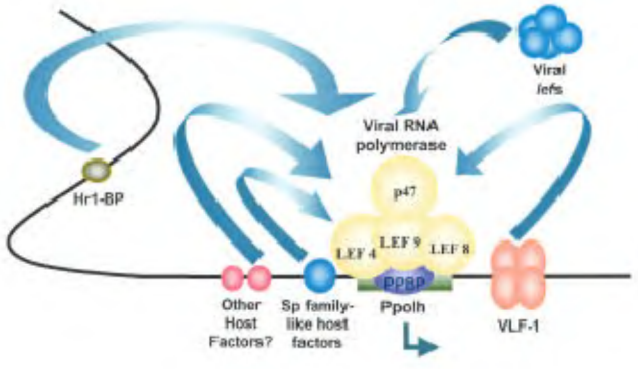

Figure 10. Regulation of transcription from the *Ac*NPV polyhedrin promoter. The hypertranscribed polh promoter recruits viral as well as insect cell host factors to enhance transcription. PPBP, which recruits the viral RNA polymerase to the transcription initiation site, Sp family-like proteins which bind to a promoter-proximal *cis*-sequence, and hr1BP, which interacts with the powerful upstream hr1 enhancer element play critical roles in this process.

regions carrying the AcSp or cSp motifs. Sp1 has been found to activate transcription from both TATA – and Inr – (initiator element) containing promoters. Further, in the bovine papillomavirus E2-responsive promoters, the TATA box or the initiator can be functionally replaced by Sp1 binding sites[98]. These data provided the first suggestion of an interaction between Sp1 and the general transcription machinery, particularly TFIID. Consistent with this observation, it was subsequently demonstrated that the human TBP-associated factor, $hTAF_{II}130$ (ref. 99), and its *Drosophila* homolog, $dTAF_{II}110$ (ref. 100), interact with the glutamine-rich activation domains of human Sp1. Thus, Sp1 is thought to function by recruiting the RNA polymerase complex to promoters via its interaction with TFIID. The most obvious explanation of the enhancement of reporter gene expression by the insect Sp family-like protein(s) is that they interact with the basal transcription machinery directly or indirectly to bring about these effects. Electromobility shift assays using the consensus TFIID oligonucleotide point to a possible interaction between the insect SP-like proteins and TFIID, hinting at a similar mode of action in *Sf*9 cells too (Ramachandran, A., unpublished observations).

Conclusion

Intensive research has gone into the elucidation of the mechanisms underlying baculovirus transcription regulation. However, the ways of the powerful BEVS remain as enigmatic as ever. An understanding of all the players involved in this process would allow us to recreate *in vitro* the conditions and factors governing polh or p10 promoters transcription, thus permitting the synthesis of foreign proteins to the desired extent while by-passing laborious tissue culture or *in vivo* systems.

In this context, host factors have emerged as a crucial component involved in regulating transcription from the baculovirus very late promoters. In addition to PPBP,

and hrBP, our observations on the presence and involvement of Sp family-like host factor(s) in insect cells is novel. Their involvement in the regulation of a gene so critical for baculovirus survival in the environment adds an important dimension to the complexity of polh promoter-driven transcription. Given that the polh promoter is a TATA-less initiator promoter, coupled with the known involvement of Sp1 in initiator-mediated transcription, these results reveal another facet of the regulation of polyhedrin-initiator transcription.

Studies are in progress to further characterize the Sp-like factor(s) which bind to AcSp and elucidate in greater detail the transcription mechanisms by which they operate. A model of the major trans-acting factor(s) influencing transcription from the polh promoter (Figure 10) thus involves an interplay of host and viral factors. Coupled with structural information and knowledge of the Sp protein(s) and other host factors' cross-talk with various cellular or viral partners, we can expect more pieces of the complex and fascinating jigsaw puzzle of baculovirus gene regulation to fall into place in the near future.

1. Mishra, S., *Curr. Sci.*, 1998, **75**, 1015–1022.
2. Kidd, M. and Emery, V. C., *Appl. Biochem. Biotechnol.*, 1993, **42**, 137–159.
3. Sridhar, P., Awasthi, A. K., Azim, C. A., Burma, S., Habib, S., Jain, A., Mukherjee, B., Ranjan, A. and Hasnain, S. E., *J. Biosci.*, 1994, **19**, 603–614.
4. O'Reilly, D. R., Miller, L. K. and Lucknow, V. A., in *Baculovirus Expression Vector: A Laboratory Manual*, W. H. Freeman and Co, New York, 1992.
5. Hasnain, S. E., Venkaiah, B. and Habib, S., US Patent #08/886,595.
6. Jha, P. K., Nakhai, B., Sridhar, P., Talwar, G. P. and Hasnain, S. E., *FEBS Lett.*, 1990, **274**, 23–26.
7. Sridhar, P. and Hasnain, S. E., *Gene*, 1993, **131**, 261–264.
8. Sridhar, P., Panda, A. K., Pal, R., Talwar, G. P. and Hasnain, S. E., *FEBS Lett.*, 1993, **315**, 282–286.
9. Mukherjee, B., Burma, S., Talwar, G. P. and Hasnain, S. E., *DNA Cell Biol.*, 1995, **14**, 7–14.
10. Ranjan, A. and Hasnain, S. E., *Indian J. Biochem. Biophys.*, 1995, **32**, 424–428.
11. Ranjan, A. and Hasnain, S. E., *Virus Genes*, 1995, **9**, 149–153.
12. Shi, Y. P., Hasnain, S. E., Sacci, J. B., Holloway, B. P., Fujioka, H., Kumar, N., Wohlhueter, R., Hoffman, S. L., Collins, W. E. and Lal, A. A., *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 1615–1620.
13. Shi, Y. P., Das, P., Holloway, B., Udhayakumar, V., Tongren, J. E., Candal, F., Biswas, S., Ahmad, R., Hasnain, S. E. and Lal, A. A., *Vaccine*, 2000, **18**, 2902–2914.
14. Hasnain, S. E., Nakhai, B., Ehtesham, N. Z., Sridhar, P., Ranjan, A., Talwar, G. P. and Jha, P. K., *DNA Cell Biol.*, 1994, **13**, 275–282.
15. Roy, P., *Ann. NY Acad. Sci.*, 1996, **791**, 318–332.
16. Chatterji, U., Ahmad, R., Venkaiah, B. and Hasnain, S. E., *Gene*, 1996, **171**, 209–213.
17. Jha, P. K., Pal, R., Nakhai, B., Sridhar, P. and Hasnain, S. E., *FEBS Lett.*, 1992, **310**, 148–152.
18. Tjia, S. T., Zu Altenschildesche, G. M. and Doerfler, W., *Virology*, 1983, **125**, 107–117.
19. Volkman, L. E. and Goldsmith, P. A., *Appl. Environ. Microbiol.*, 1983, **45**, 1085–1093.
20. Boyce, F. M. and Bucher, N. L., *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 2348–2352.
21. Hofmann, C., Sandig, V., Jennings, G., Rudolph, M., Schlag, P. and Strauss, M., *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 10099–10103.
22. Ghosh, S., Parvez, M. K., Banerjee, K., Sarin, S. K. and Hasnain, S. E., 2001, manuscript submitted.
23. Gronowski, A. M., Hilbert, D. M., Sheehan, K. C., Garotta, G. and Schreiber, R. D., *J. Virol.*, 1999, **73**, 9944–9951.
24. Bashyam, M. D., Ramachandran, A., Ghosh, S., Viswanathan, P. and Hasnain, S. E., *Proc. Indian Natl. Sci. Acad.*, 2000, **B66**, 83–96.
25. Carson, D. D., Guarino, L. A. and Summers, M. D., *Virology*, 1988, **162**, 144–151.
26. Carson, D. D., Summers, M. D. and Guarino, L. A., *Virology*, 1991, **182**, 279–286.
27. Guarino, L. A. and Summers, M. D., *J. Virol.*, 1986, **57**, 563–571.
28. Guarino, L. A. and Summers, M. D., *J. Virol.*, 1987, **61**, 2091–2099.
29. Guarino, L. A. and Summers, M. D., *J. Virol.*, 1988, **62**, 463–471.
30. Blissard, G. W. and Rohrmann, G. G., *Annu. Rev. Entomol.*, 1990, **35**, 127–155.
31. Chaeychomsri, S., Ikeda, M. and Kobayashi, M., *Virology*, 1995, **206**, 435–447.
32. Huh, N. E. and Weaver, R. F., *J. Gen. Virol.*, 1990, **71**, 195–202.
33. Chisholm, G. E. and Henner, D. J., *J. Virol.*, 1988, **62**, 3193–3200.
34. Krappa, R. and Knebel-Morsdorf, D., *J. Virol.*, 1991, **65**, 805–812.
35. Leisy, D. J., Rasmussen, C., Kim, H.-T. and Rohrmann, G. F., *Virology*, 1995, **208**, 742–752.
36. Lu, A. and Carstens, E. B., *Virology*, 1993, **195**, 710–718.
37. Habib, S. and Hasnain, S. E., *J. Biol. Chem.*, 1996, **271**, 28250–28258.
38. Kool, M., Ahrens, C. H., Vlak, J. M. and Rohrmann, G. R., *J. Gen. Virol.*, 1995, **76**, 2103–2118.
39. Pearson, M., Bjornson, R., Pearson, G. and Rohrmann, G., *Science*, 1992, **257**, 1382–1384.
40. Habib, S., Pandey, S., Chatterji, U., Burma, S., Ahmad, R., Jain, A. and Hasnain, S. E., *DNA Cell Biol.*, 1996, **15**, 737–747.
41. Habib, S. and Hasnain, S. E., *J. Virol.*, 2000, **74**, 5182–5189.
42. Leisy, D. J., Rasmussen, C., Owusu, E. O. and Rohrmann, G. F., *J. Virol.*, 1997, **71**, 5088–5094.
43. Murges, D., Kremer, A. and Knebel-Morsdorf, D., *J. Gen. Virol.*, 1997, **78**, 1507–1510.
44. Kovacs, G. R., Guarino, L. A. and Summers, M. D., *J. Virol.*, 1991, **65**, 5281–5288.
45. Yoo, S. and Guarino, L. A., *Virology*, 1994, **202**, 746–753.
46. Yoo, S. and Guarino, L. A., *Virology*, 1994, **202**, 164–172.
47. Knebel-Morsdorf, D., Kremer, A. and Jahnel, F., *J. Virol.*, 1993, **67**, 753–758.
48. Thiem, S. M. and Miller, L. K., *J. Virol.*, 1989, **63**, 4489–4497.
49. Clem, R. J. and Miller, L. K., *J. Virol.*, 1993, **67**, 3730–3788.
50. Hershberger, P. A., Dickson, J. A. and Friesen, P. D., *J. Virol.*, 1992, **66**, 5525–5533.
51. Hasnain, S. E., Taneja, T. K., Sah, N. K., Mohan, M., Pathak, N., Sahdev, S., Athar, M. and Begum, R., *J. Biosci.*, 1999, **24**, 101–107.

52. Sah, N. K., Taneja, T. K., Pathak, N., Begum, R., Athar, M. and Hasnain, S. E., *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 4838–4843.
53. Kool, M., Ahrens, C., Goldbach, R. W., Rohrmann, G. F. and Vlak, J. M., *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 11212–11216.
54. Lu, A. and Miller, L. K., *J. Virol.*, 1995, **69**, 975–982.
55. Lu, A. and Carstens, E. B., *Virology*, 1991, **181**, 336–347.
56. Croizier, G., Croizier, L., Argaud, O. and Poudevigne, D., *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 48–52.
57. Laufs, S., Lu, A., Arrell, L. K. and Carstens, E. B., *Virology*, 1997, **228**, 98–106.
58. Liu, G. and Carstens, E. B., *Virology*, 1999, **253**, 125–136.
59. McDougal, V. V. and Guarino, L. A., *J. Virol.*, 2000, **74**, 5273–5279.
60. McDougal, V. V. and Guarino, L. A., *J. Virol.*, 2001, **75**, 7206–7209.
61. Wu, Y. and Carstens, E. B., *Virology*, 1998, **247**, 32–40.
62. Gong, M., Jin, J. and Guarino, L. A., *Virology*, 1998, **244**, 495–503.
63. Guarino, L. A., Xu, B., Jin, J. and Dong, W., *J. Virol.*, 1998, **72**, 7985–7991.
64. Beniya, H., Joel, Funk, C., Rohrmann, G. F. and Weaver, R. F., *Virology*, 1996, **216**, 12–19.
65. Glocker, B., Hoopes, R. R. and Rohrmann, G. F., *J. Virol.*, 1993, **67**, 3771–3776.
66. Yang, C. L., Stetler, D. A. and Weaver, R. F., *Virus Res.*, 1991, **20**, 251–264.
67. Morris, T. D. and Miller, L. K., *Gene*, 1994, **140**, 147–153.
68. Ooi, B. G., Rankin, C. B. and Miller, L. K., *J. Mol. Biol.*, 1989, **210**, 721–736.
69. Weyer, U. and Possee, R. D., *J. Gen. Virol.*, 1989, **70**, 203–208.
70. Todd, J. W., Passarelli, A. L., Lu, A. and Miller, L. K., *J. Virol.*, 1996, **70**, 2307–2317.
71. Yang, S. and Miller, L. K., *Virology*, 1998, **248**, 131–138.
72. Yang, S. and Miller, L. K., *Virology*, 1998, **245**, 99–109.
73. Yang, S. and Miller, L. K., *J. Virol.*, 1999, **73**, 3404–3409.
74. Lu, A. and Miller, L. K., *J. Virol.*, 1994, **68**, 6710–6718.
75. Morris, T. D., Todd, J. W., Fisher, B. and Miller, L. K., *Virology*, 1994, **200**, 360–369.
76. Passarelli, A. L. and Miller, L. K., *J. Virol.*, 1994, **68**, 1186–1190.
77. Passarelli, A. L., Todd, J. W. and Miller, L. K., *J. Virol.*, 1994, **68**, 4673–4678.
78. Hang, S., Dong, W. and Guarino, L. A., *J. Virol.*, 1995, **69**, 3924–3928.
79. Evans, J. T. and Rohrmann, G. F., *J. Virol.*, 1997, **71**, 3574–3579.
80. Evans, J. T., Leisy, D. J. and Rohrmann, G. F., *J. Virol.*, 1997, **71**, 3114–3119.
81. Merrington, C. L., Kitts, P. A., King, L. A. and Possee, R. D., *Virology*, 1996, **217**, 338–348.
82. Guarino, L. A., Dong, W., Xu, B., Broussard, D. R., Davis, R. W. and Jarvis, D. L., *J. Virol.*, 1992, **66**, 7113–7120.
83. Jin, J., Ding, W. and Guarino, L. A., *J. Virol.*, 1998, **72**, 10011–10019.
84. Harwood, S. H., Li, L., Ho, P. S., Preston, A. K. and Rohrmann, G. F., *Virology*, 1998, **250**, 118–134.
85. Rapp, J. C., Wilson, J. A. and Miller, L. K., *Virology*, 1998, **72**, 10197–10206.
86. Hasnain, S. E., Jain, A., Habib, S., Ghosh, S., Chatterji, U., Ramachandran, A., Das, P., Venkaiah, B., Pandey, S., Liang, B., Ranjan, A., Natarajan, K. and Azim, C. A., *Gene*, 1997, **190**, 113–118.
87. Etkin, E., Carp-Weiss, L. and Levi, B. Z., *Virus Res.*, 1994, **31**, 343–349.
88. Burma, S., Mukherjee, B., Jain, A., Habib, S. and Hasnain, S. E., *J. Biol. Chem.*, 1994, **269**, 2750–2757.
89. Hasnain, S. E., Habib, S., Jain, A., Burma, S. and Mukherjee, B., *Methods Enzymol.*, 1996, **274**, 20–32.
90. Ghosh, S., Jain, A., Mukherjee, B., Habib, S. and Hasnain, S. E., *J. Virol.*, 1998, **72**, 7484–7493.
91. Mukherjee, B., Burma, S. and Hasnain, S. E., *J. Biol. Chem.*, 1995, **270**, 4405–4411.
92. Jain, A. and Hasnain, S. E., *Eur. J. Biochem.*, 1996, **239**, 384–390.
93. Ahrens, C. H., Leisy, D. J. and Rohrmann, G. F., in *Baculovirus DNA Replication* (ed. De Pamphilis, M.), CSHL Press, Cold Spring Harbor, New York, 1996.
94. Possee, R. D. and Rohrmann, G. F., Baculovirus genome organization and evolution, in *The Baculoviruses* (ed. Miller, L. K.), Plenum, New York, 1997, pp. 109–138.
95. Ramachandran, A., Jain, A., Arora, P., Bashyam, M. D., Chatterjee, U., Ghosh, S., Parnaik, V. K. and Hasnain, S. E., *J. Biol. Chem.*, 2001, **276**, 23440–23449.
96. Turner, J. and Crossley, M., *Trends Biochem. Sci.*, 1999, **24**, 236–241.
97. Philipsen, S. and Suske, G., *Neucleic Acids Res.*, 1999, **27**, 2991–3000.
98. Ham, J., Steger, G. and Yaniv, M., *EMBO J.*, 1994, **13**, 147–157.
99. Tanese, N., Saluja, D., Vassallo, M. F., Chen, J.-L. and Admon, A., *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 13611–13616.
100. Hoey, T., Weinzierl, R. O., Gill, G., Chen, J.-L., Dynlacht, B. D. and Tjian, R., *Cell*, 1993, **72**, 247–260.

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