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Identification of an enhancer-like element in the polyhedrin gene upstream region of *Bombyx mori* nucleopolyhedrovirus

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A series of deletions in the upstream region of the gene encoding polyhedrin (*polh*) of *Bombyx mori* nucleopolyhedrovirus (BmNPV) were generated in plasmid constructs and tested for transcription. In transient transfection assays in *Bombyx mori*-derived BmN cells with firefly luciferase as the reporter gene, a 293 bp fragment located 1·0 kb upstream with respect to the +1 ATG of *polh* showed 10-fold enhancement in expression from the minimal promoter. This increase in reporter activity was observed only when the fragment was positioned *in cis* with respect to the promoter and not *in trans*. The stimulation of reporter gene expression was independent of the orientation of the fragment and was due to increased transcription from the promoter. When placed upstream of another promoter, the viral very late gene *p10* promoter, the enhancer brought about a 2-fold increase in expression. The region encompassing the enhancer was itself transcriptionally active, and transcripts corresponding to both of the encoded ORFs (N-terminal regions of ORF453 and ORF327, located in opposite orientations) were detected. Two AP1 sites (TGACTCG) in the 293 bp fragment did not appear to contribute to the enhancer function. Since repeat motifs, the hallmark of conventional enhancer sequences, were absent from this fragment, it is designated as an enhancer-like element. The influence of this region of the *polh* upstream sequence on expression from strong, very late viral promoters has not been reported previously.

Introduction

The baculoviruses comprise a large family of occluded DNA viruses with covalently closed, circular, double-stranded genomes that primarily infect the holometabolous insects (Blissard & Rohrmann, 1990; Hayakawa *et al.*, 2000). Among these, *Autographa californica* multinucleocapsid nucleopolyhedro virus (AcMNPV), a virus of the alfalfa looper, is by far the best studied at the molecular level and hence serves as the prototype baculovirus. Parallel investigations of several other baculoviruses, such as *Bombyx mori* nucleopolyhedrovirus (BmNPV), *Lymantria dispar* MNPV (LdMNPV) and *Orgyia pseudotsugata* MNPV (OpMNPV), have been carried out and their full genomic sequences have been reported (Ayres *et al.*, 1994; Gomi *et al.*, 1999; Kuzio *et al.*, 1999; Ahrens *et al.*, 1997). A distinctive feature of the baculovirus life cycle is the synthesis of two forms of infectious particles, which is temporally regulated via a cascade of gene expression pathways (Keddie *et al.*, 1989). The budded or extracellular virus is released from the cell between 8 and 12 h post-infection (p.i.) and requires viral DNA replication and synthesis of structural proteins. As the infection proceeds into the very late phase (20–72 h p.i.), the virions are occluded into large polyhedral bodies inside the nucleus. A 29 kDa polypeptide known as polyhedrin constitutes the major component of these occlusion bodies. Polyhedrin, despite being non-essential for virus propagation *in vitro*, is produced abundantly at late times. Other very late, non-essential genes like *p10* are also expressed to very high levels. The occluded virion can survive adverse environmental conditions and has evolved to mediate horizontal transmission of the pathogen. The replacement of the gene encoding polyhedrin (*polh*) with a foreign gene of interest to generate recombinant viruses has formed the basis of the baculovirus-based overexpression systems. The analysis of regulation of expression from this promoter is therefore significant.

An interesting feature of the late and very late viral promoters is that transcription from these promoters is carried out by an RNA polymerase distinct from the host-cell polymerases, made up entirely of four virus-encoded subunits, LEF8, LEF9, LEF4 and p47 (Guarino *et al.*, 1998). The AcMNPV *polh* promoter has been studied extensively by using both deletion (Possee & Howard, 1987) and linker-scan

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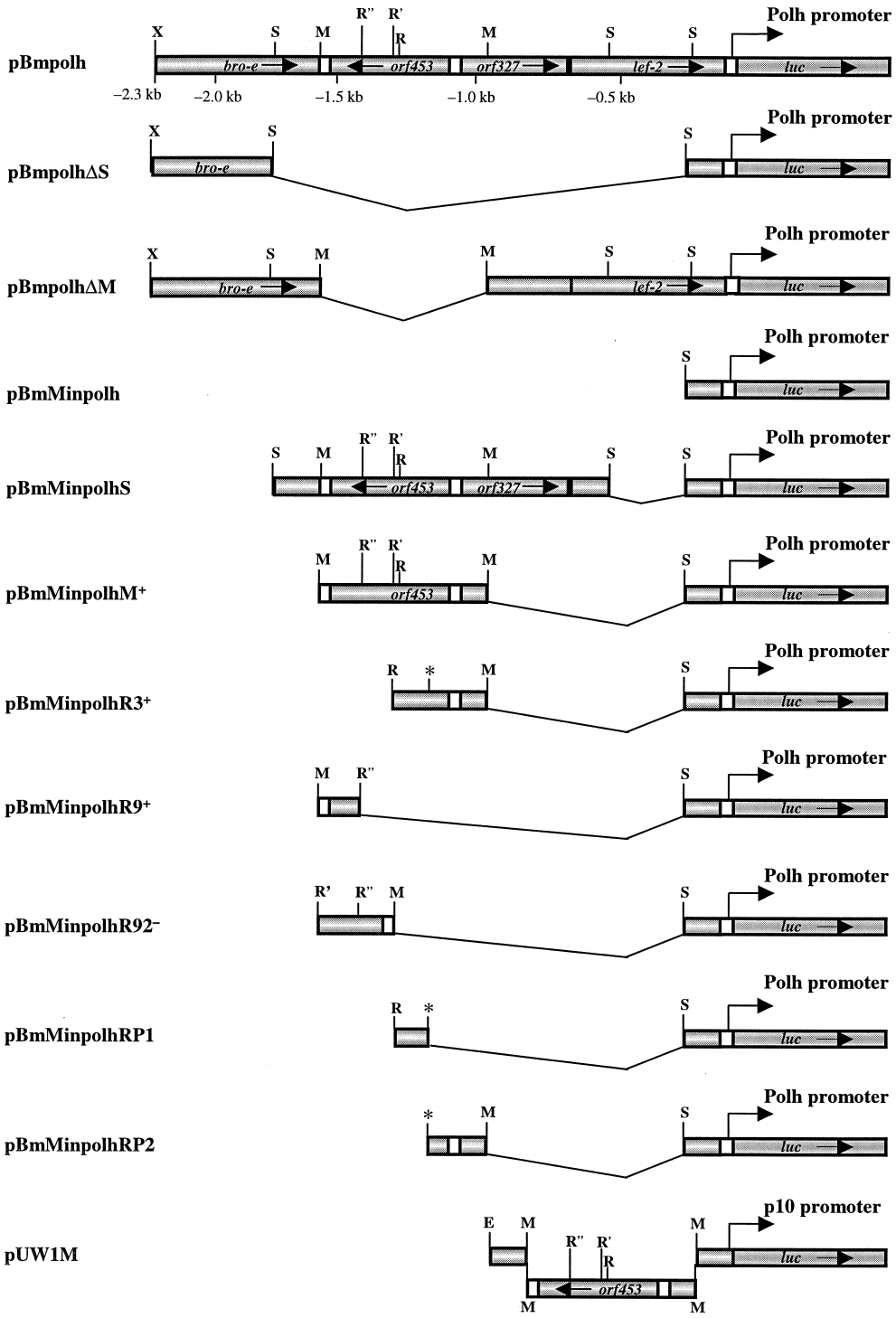

Fig. 1. Plasmid constructs generated in the study. The plasmid constructs generated that harbour different lengths of the BmNPV *polh* 5′ upstream region are shown schematically. The reporter luciferase gene (*luc*), containing an 80 bp leader sequence at the 5′ end and the polyadenylation signals at the 3′ end, was placed immediately downstream of the *polh* promoter (cloning site at −3 nt with respect to +1 ATG of polyhedrin and 50 nt downstream of the TAAG transcription start site motif). Plasmid construction was carried out essentially as described in Sambrook *et al.* (1989). For details of the individual plasmid constructs, see Methods. The 3′ and 5′ ends of the different constructs shown extend into the vector pBS KS$^+$ sequences. The symbols + and − indicate the presence of the insert in one or other orientation, while * indicates the primer site used in generation of the PCR subclones of the 293 bp *Rsa*I–*Mlu*I fragment. The 101 bp (R–*) fragment harboured two AP1 sites and the 198 bp (*–M) fragment harboured one AP1 site. Restriction sites indicated are: E, *Eco*RI; M, *Mlu*I; R, R′ and R″, *Rsa*I; S, *Sal*I; and X, *Xho*I.

(Rankin *et al.*, 1988; Ooi *et al.*, 1989) mutational analysis. These studies identified a conserved TAAG core sequence that serves both as an integral promoter element and as the transcription start point. Besides the core sequence, linker-scan studies also demonstrated that the region between the core sequence and the translational initiation site greatly affects the steady-state level of *polh* transcript (Ooi *et al.*, 1989). However, in addition to these proximal promoter elements, *polh* transcription is also influenced by distal enhancer elements in AcMNPV. Interspersed throughout the length of the baculovirus genome are homologous repeat (*hr*) sequences that are composed of several (two to eight) ~ 30 bp imperfect palindromic sequences. Transient *in vitro* plasmid-based replication assays have shown that the *hr* sequences function as origins of viral DNA replication (Pearson *et al.*, 1992; Leisy & Rohrmann, 1993). The *hr* sequences have also been shown to act as enhancers. For instance, the AcMNPV *hr*5 functions as a transcriptional enhancer of the delayed-early gene *39K* (Guarino & Summers, 1986) and the transactivator protein IE-1 is a component of the DNA–protein complexes in this *hr*5-mediated enhancer function (Guarino & Dong, 1994). The AcMNPV *hr*1 enhances reporter expression from the *polh* promoter (Habib *et al.*, 1996). Likewise, the BmNPV *hr*3 acts as an enhancer for the *B. mori* cytoplasmic actin gene promoter *in vitro*, augmenting reporter expression in transfected cells by two orders of magnitude (Lu *et al.*, 1997). This was stimulated further more than 1000-fold on supplementation of transfected cells with the BmNPV transactivator protein IE-1.

In an attempt to characterize further the *polh* promoter of BmNPV, we have analysed the far-upstream region, beyond the core sequence element. A comparison of this region between the genomes of AcMNPV (Ayres *et al.*, 1994) and BmNPV (Gomi *et al.*, 1999) revealed an overall conservation of sequences as well as some marked differences. Starting with a reporter plasmid construct containing as much as 2·3 kb of the upstream region of the *polh* promoter, a series of deletions were generated. Using the minimal *polh* promoter construct harbouring the core sequence TAAG as the reference plasmid, our studies identified a 293 bp fragment located approximately 1·0 kb upstream of the promoter that enhanced transcription from the minimal *polh* promoter over 10-fold in a position- and orientation-independent fashion. This region, however, encoded the N-terminal segments of ORF453 and ORF327, located in opposite orientations, and both were actively transcribed.

Methods

■ **Generation of plasmid constructs.** The recombinant plasmids generated in the study are presented in Fig. 1. A 2·3 kb *Xho*I–*Eco*RI fragment corresponding to the BmNPV genomic sequences of the *polh* upstream region was mobilized from the plasmid pBm030 (Maeda, 1989) into pBS KS+. The reporter gene, firefly luciferase (*luc*; a 1·8 kb DNA fragment containing an 80 bp untranslated leader sequence as well as a polyadenylation signal at the 3′ end) was cloned downstream of the promoter as a *Bam*HI fragment to generate the construct pBmpolh. A minimal promoter construct, pBmMinpolh, containing 192 bp of the *polh* immediate 5′ upstream sequences and encompassing the consensus core promoter motif TAAG, was made by subcloning the PCR-amplified *Sal*I–*Eco*RI fragment of pBm030 in pBS KS+ and inserting *luc* downstream of the promoter. From the parental construct, pBmpolh, deletion of a 1·6 kb *Sal*I fragment followed by self-ligation generated pBmpolhΔS, while deletion of a 665 bp *Mlu*I fragment gave rise to the construct pBmpolhΔM. A 1·2 kb *Sal*I fragment and the 665 bp *Mlu*I fragment were mobilized separately into the *Sal*I site in pBmMinpolh to generate pBmMinpolhS and pBmMinpolhM, respectively. The *Mlu*I fragment was cloned in both orientations with respect to the *polh* promoter (after blunt-ending both the vector and the insert with Klenow DNA polymerase) to provide pBmMinpolhM$^{+/-}$.

Subclones of the 665 bp *Mlu*I fragment were made by partial digestion of this fragment with *Rsa*I and cloning the digested fragments at the *Sal*I site of pBmMinpolh after blunt-ending with Klenow DNA polymerase. The full fragment was represented in four subclones generated in this way, pBmMinpolhR92^{-}, pBmMinpolhR9^{+}, pBmMinpolhR3^{+} and pBmMinpolhR3^{-}. pBmMinpolhR92^{-} contained 188 and 114 bp fragments in inverse orientations while pBmMinpolhR9^{+} had only the 188 bp fragment in the proper orientation. Plasmids pBmMinpolhR3^{+} and pBmMinpolhR3^{-} contained the 293 bp *Rsa*I–*Mlu*I fragment cloned in both orientations. This fragment was split further into two fragments of 101 bp and 198 bp by PCR amplification (using primers P1a, 5′ GCGTCGACGCTTGACTCGGG 3′, and P1b, 5′ GCGTCGACGTACATCCTCGTTT 3′, for the 101 bp fragment and P2a, 5′ GCGTCGACGCGTGCACATATC 3′, and P2b, 5′ GCGTCGACCCGAGTCAAGCGCAG 3′, for the 198 bp fragment) and subcloned individually in pBmMinpolh to generate pBmMinpolhRP1 and pBmMinpolhRP2, respectively. In order to analyse the influence of the *Mlu*I fragment on transcription from other promoters, it was mobilized upstream of the AcMNPV *p10* promoter in the plasmid construct pUW1 (Sriram *et al.*, 1997), generating pUW1M. Yet another plasmid construct, pTZSt, harbouring the 1·2 kb *Sal*I fragment (encompassing ORF453, ORF327 and the N-terminal half of LEF-2) in pTZ18R, was generated in order to analyse its effect on expression from the *polh* promoter *in trans* (this construct is not shown in Fig. 1).

■ **Cell culture and transfections.** The *B. mori*-derived cell line BmN was maintained at 27 °C in TC-100 medium supplemented with 10% foetal bovine serum (Gibco BRL). Transfections with various plasmid DNA constructs were carried out in 6-well microtitre plates (10^6 cells per well in 2 ml medium). The cells were infected with wild-type BmNPV at an m.o.i. of 10 for all the transient transfections (Palhan *et al.*, 1995). The virus stocks were maintained and titres were determined according to standard protocols (O'Reilly *et al.*, 1992). The cells were transfected with plasmid constructs (2·5 μg covalently closed, circular DNA) using lipofectin (Gibco BRL) in serum-free medium for 8 h, followed by virus infection at an m.o.i. of 10 (Palhan *et al.*, 1995). The cells were incubated at 27 °C for 48 h, harvested and washed with PBS (10 mM KH_2PO_4, 2 mM Na_2HPO_4, 140 mM NaCl and 25 mM KCl). Luciferase activity in the cells was quantified (non-invasive assay) by using a luminometer (Palhan *et al.*, 1995; Sriram *et al.*, 1997).

■ **RNA isolation and slot-blot hybridization.** Total RNA was isolated from transfected cells by the guanidinium isothiocyanate method (Chomczynski & Sacchi, 1987) and treated with RNase-free DNase. RNA slot-blot analysis was carried out with 5 μg total RNA in 50% formamide, 6% formaldehyde and 1 × SSC (0·15 M NaCl and 0·1 M sodium citrate, pH 7·4), blotted on to a nylon membrane (Amersham) and probed using radiolabelled *luc* and tRNA probes. Hybridization was carried out at

42 °C in the presence of 50% formamide and the blots were washed at a final stringency of 0·1 × SSC and 0·1% SDS at 65 °C for 30 min.

■ **RNase protection assays.** The transcription status of ORF453 and ORF327 was analysed by an RNase protection assay using corresponding antisense RNA probes. For ORF453, the plasmid pBmMinpolhR3^{+}, encompassing the N-terminal region of the gene, was linearized by digestion with *Mlu*I and transcribed *in vitro* using T3 RNA polymerase in the presence of radiolabelled [α-^{32}P]UTP. The ^{32}P-labelled probe for ORF327 was generated similarly from plasmid pBmMinpolhR3^{-}. The antisense riboprobes ($1{\cdot}5 \times 10^{5}$ c.p.m.) were co-precipitated with 20 μg total RNA, isolated from both uninfected and infected cells at various times p.i. in the presence of 200 mM NaCl and 20 μg carrier RNA using 2·5 vols ethanol. Following hybridization at 50 °C overnight in the presence of 50% formamide, an RNase digestion mixture containing RNase A (2 U) and RNase T1 (1 U) was added and the samples were precipitated in the presence of 10 μg yeast tRNA and 2·5 vols ethanol. The RNase-protected samples were analysed by electrophoresis on 8 M urea–6% acrylamide gels and visualized by autoradiography.

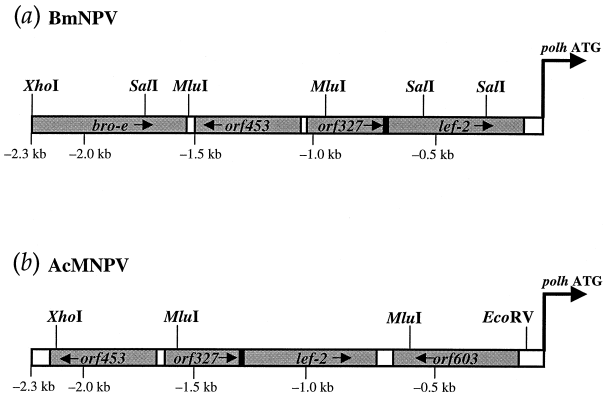

Fig. 2. Comparison of the *polh* upstream regions of AcMNPV and BmNPV. The 2·3 kb *polh* upstream regions (98·2–100 map units) of BmNPV (*a*) and AcMNPV (*b*) are aligned. Major ORFs are represented as boxes at appropriate positions and their orientations are indicated by arrows. The locations of restriction sites and the *polh* + 1 ATG are indicated. ORF453, ORF327, *lef-2* and ORF603 correspond to AcMNPV ORFs 4, 5, 6 and 7, respectively. The overlap between ORF327 and *lef-2* is indicated as a shaded region. The *bro-e* sequence at this location is exclusive to BmNPV, whereas ORF603 (Ac7) is absent in BmNPV.

Results

Analysis of the 5′ flanking region of *polh*

We have analysed the effect of the 5′ upstream region of *polh* on transcription from the *polh* promoter. The AcMNPV and BmNPV promoters function equally well in the host cells Sf21 and BmN on infection with AcMNPV and BmNPV, respectively (Palhan *et al.*, 1995; Sriram *et al.*, 1997). A comparison of the genomic regions up to 2·3 kb upstream of the *polh* promoter of AcMNPV and BmNPV is presented in Fig. 2. This region encompasses AcMNPV ORFs 4–7, of which Ac4, Ac5 and Ac6 show greater than 92% identity to BmNPV counterparts at the amino acid level. Ac6, commonly referred to as *lef-2* (late gene expression factor-2), is essential for both viral DNA replication and late gene expression in AcMNPV, BmNPV and OpMNPV (Merrington *et al.*, 1996; Sriram & Gopinathan, 1998; Ahrens & Rohrmann, 1995; Sriram, 1998). The functions of ORF453 (Ac4) and ORF327 (Ac5) remain unidentified in all the baculoviruses characterized so far. The major differences were in ORF603 (Ac7) and the *bro-e* gene of BmNPV (Fig. 2). ORF603, located immediately upstream of the *polh* promoter in AcMNPV, is absent from the BmNPV genome (Gomi *et al.*, 1999). This ORF is a non-essential gene in AcMNPV, since its disruption had no effect on virus infectivity or polyhedron production (Gearing & Possee, 1990). The gene designated *bro-e*, present at about 2·0 kb upstream in BmNPV, is absent in AcMNPV and belongs to the highly repeated baculovirus-repeated ORF (*bro*) gene family (Kang *et al.*, 1999). There are five copies of *bro* (*bro a–e*) in BmNPV, whereas AcMNPV encodes only a single copy (Ac2, a homologue of BmNPV *bro-d*) that is located elsewhere in its genome. The encoded products of *bro* genes have been shown to bind nucleic acids and are involved in nucleosome organization (Zemskov *et al.*, 2000). The presence of *bro-e* in the upstream region of *polh* of BmNPV compensates for the absence of ORF603 to locate *hr*1, which has an enhancer function (see the following section), at the same position with respect to the *polh* + 1 ATG in both the viruses.

A 293 bp fragment from the *polh* upstream region enhances expression

In order to achieve the high levels of expression observed from the AcMNPV promoter, the virus *hr*1 sequences located about 4 kb upstream of the *polh* + 1 ATG site have been shown to function as an enhancer (Habib *et al.*, 1996). The BmNPV *polh* upstream region also harbours a similar *hr* sequence at the same distance (4·2 kb). In order to study the effect of *cis* elements other than the *hr* sequence from the *polh* upstream region, we generated a series of plasmid constructs harbouring *luc* as the reporter placed under the *polh* promoter covering different regions of the 5′ upstream sequence, up to 2·3 kb (Fig. 1). The parental plasmid construct pBmpolh, harbouring the entire 2·3 kb 5′ sequence of the BmNPV T3 strain (nt 126087–128413 on the BmNPV genome, GenBank accession no. L33180), was derived from the transfer vector pBm030 (Maeda, 1989). The minimal *polh* promoter plasmid (pBmMinpolh), containing up to 192 nt upstream of the *polh* ATG, was used as the reference to study the influence of other regulatory elements. Reporter gene (*luc*) expression was 25-fold higher compared with the minimal promoter construct (Fig. 3) when the additional 2·1 kb was added (plasmid pBmpolh). Deletion of a 1·6 kb *Sal*I fragment (nt 126625–128227) that removed the whole of ORF327, ORF453 and parts of the *lef-2* and *bro-e* sequences (plasmid pBmpolhΔS) reduced reporter expression to the same level as that of pBmMinpolh, suggesting the presence of a positive regulatory element in this region.

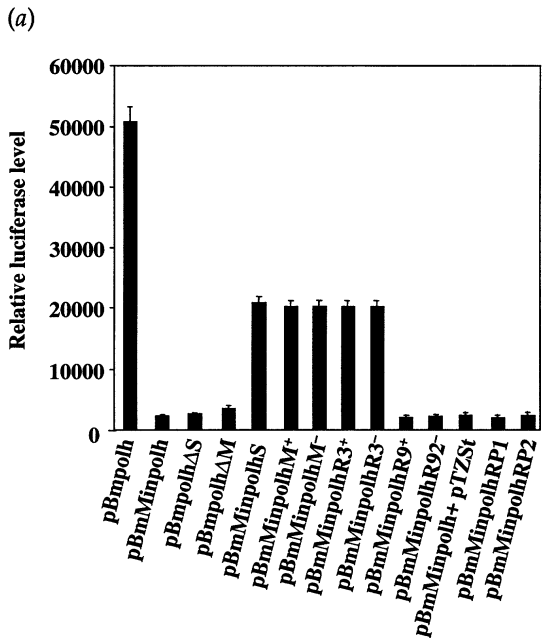

Fig. 3. Relative luciferase levels from varying lengths of the *polh* upstream region. Different plasmid constructs (2·5 μg DNA) were transfected into BmN cells by lipofection (Palhan *et al.*, 1995) and, after 8 h at 27 °C, the cells were infected with BmNPV (m.o.i. of 10). At 48 h p.i., the cells were harvested, washed in PBS and resuspended in luciferase assay buffer. For the luciferase assay (non-invasive method), 10% of the cell suspension was added to the assay buffer in the presence of 10 μM luciferin at pH 7·2 and light emission was monitored in a luminometer. (*a*) Activity expressed as relative light units per μg protein for various constructs of the *polh* promoter. (*b*) Expression levels of luciferase from the *p10* promoter with (left) and without (right) the 665 bp enhancer fragment *in cis*. The luciferase activity levels are means of three independent transfection experiments.

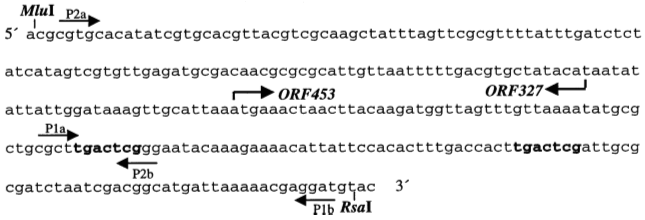

Fig. 4. Sequence of the enhancer-harbouring *Mlu*I–*Rsa*I fragment. The nucleotide sequence is presented of a 293 bp fragment (nt 127464–127168) on the BmNPV genome that shows enhancer activity. Locations of ORF327 and ORF453, in opposite orientations, are indicated and the two AP1 sites identified are shown in bold. P1a, P1b, P2a and P2b indicate the locations and directions of the primers used to generate the constructs pBmMinpolhRP1 and pBmMinpolhRP2.

This reduction in activity was not due to the deletion of *lef-2*, as the following studies show. When the 665 bp *Mlu*I fragment (nt 126799–127464) was deleted from pBmpolh to give construct pBmpolhΔM (Fig. 1), despite the presence of intact *lef-2*, luciferase levels were comparable to those from the minimal promoter alone (Fig. 3). Furthermore, LEF-2 was provided *in trans* in all instances by virus infection following transfection. These results implied that the positive element present within the 1·6 kb *Sal*I region was confined to the 665 bp *Mlu*I fragment. To confirm this, the 1·2 kb *Sal*I fragment and 665 bp *Mlu*I fragment were reintroduced separately into the minimal *polh* promoter construct to generate pBmMinpolhS and pBmMinpolhM, respectively (Fig. 1). They showed equal stimulation in reporter gene activity from pBmMinpolh (Fig. 3). The extent of stimulation was similar with the *Mlu*I fragment inserted in either orientation (pBmMinpolhM$^{+/-}$), suggesting that the DNA fragment behaved like an enhancer element. The extent of stimulation, however, was limited to 10-fold, compared with the 25-fold stimulation seen with pBmpolh (harbouring the entire 2·3 kb upstream sequence). Since the *Mlu*I fragment encompassed the complete ORF453 together with its 150 bp upstream region (with respect to its +1 ATG) as well as the N-terminal region of ORF327, the possibility of activation *in trans* by the corresponding encoded proteins could not be ruled out. To test this, plasmid pTZSt (harbouring the 1·2 kb *Sal*I fragment with both the ORFs cloned in plasmid pTZ18R) was co-transfected together with minimal promoter or pBmpolhΔM construct. No stimulation of the luciferase activity was seen (Fig. 3), ruling out *trans*-activation.

In order to narrow down the *cis*-acting element further, the 665 bp *Mlu*I region was partially digested with *Rsa*I and the individual fragments were cloned into pBmMinpolh to generate four additional subclones, pBmMinpolhR9^{+}, pBmMinpolhR92^{-}, pBmMinpolhR3^{+} and pBmMinpolhR3^{-} (Fig. 1). pBmMinpolhR9^{+} had a 188 bp *Rsa*I–*Mlu*I fragment (nt 126800–126988 on the BmNPV genome) cloned in the right orientation whereas pBmMinpolhR92^{-} carried the same 188 bp fragment as well as the 114 bp fragment (nt 126800–127102) in the inverse orientation. The 293 bp *Rsa*I–*Mlu*I region (nt 127170–127463), encompassing the N-terminal regions of ORF453 and ORF327, was cloned in both orientations, giving pBmMinpolhR3^{+} and pBmMinpolhR3^{-}. When the above constructs were transfected into BmN cells, pBmMinpolhR9^{+} and pBmMinpolhR92^{-} failed to activate *luc* expression. On the other hand, both plasmids pBmMinpolhR3^{+} and pBmMinpolhR3^{-} stimulated expression from the minimal *polh* promoter to the same extent as the

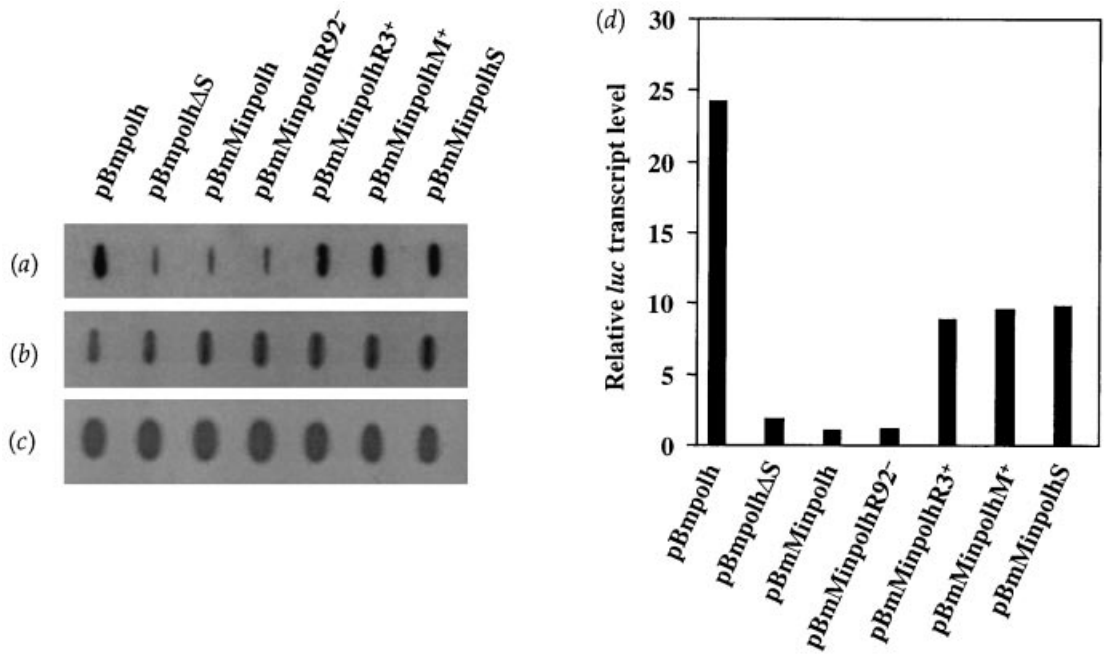

Fig. 5. Quantification of transcripts from the enhancer-containing plasmids. The reporter gene (*luc*) transcript from the transfected cells was quantified by RNA slot blots. Total RNA was isolated from BmN cells transfected with plasmid DNA (2·5 μg each) from pBmpolh (parental construct), pBmpolhΔS, pBmMinpolh, pBmMinpolhR92^{-} or the enhancer-harbouring constructs pBmMinpolhS, pBmMinpolhM^{+} and pBmMinpolhR3^{+}, followed by BmNPV infection (m.o.i. of 10). The cells were harvested at 48 h p.i. and RNA samples (5 μg each) were blotted on to nylon membranes. Hybridization was carried out at 42 °C for 12 h using a radiolabelled *luc* probe generated by random priming of the 1·8 kb *luc* DNA by Klenow DNA polymerase in presence of [α-^{32}P]dATP. The membranes were washed with 0·1 × SSC and 0·1 % SDS at 65 °C and subjected to autoradiography (*a*). Subsequently, the membranes were stripped and reprobed with labelled tRNA$_1^{Gly}$ probe to normalize to endogenous transcript levels as a control for RNA loading (*b*). DNA was also extracted from the transfected samples and was probed with radiolabelled plasmid pBS KS^{+} DNA to normalize the levels of transfected DNA (*c*). (*d*) Quantification of RNA transcripts by densitometric scanning following autoradiography. The *luc* transcript levels were normalized to the levels of endogenous tRNA transcripts (*a*/*b*) to correct for any loading artefacts between the samples and these transcript levels were converted to levels per copy of the transfected plasmid DNA [(*a*/*b*)/*c*].

parental construct pBmMinpolhM (Fig. 3). This activation was thus independent of the orientation of the insert, further substantiating the enhancer model.

Since the 293 bp fragment contained two AP1 sequence motifs, we examined whether the enhancing effect of this fragment on transcription from the *polh* promoter was due to the presence of these motifs. For this purpose, the 293 bp region was split into two fragments, of 101 bp (nt 127168–127269 on the BmNPV genome) and 198 bp (nt 127256–127464), with an overlap of 16 nt between them. The 101 bp fragment contained two AP1 sequence motifs (TGACTCG) separated by 40 nt, whereas the 198 bp fragment retained only one of the AP1 sites, at the extreme 5′ end of the fragment. These two fragments were generated by PCR amplification from genomic DNA using appropriate primers and cloned individually into pBmMinpolh to give constructs pBmMinpolhRP1 and pBmMinpolhRP2 (harbouring the 101 and 198 bp fragments, respectively; Fig. 1). Neither of the constructs showed stimulation of luciferase expression over and above the minimal promoter construct. The minimal enhancer element could thus be narrowed down only to the 293 bp *Rsa*I–*Mlu*I fragment presented in Fig. 4.

The increase in *luc* expression was due to enhanced transcription

In order to determine whether the increase observed in luciferase activity in the presence of the *Rsa*I–*Mlu*I fragment was indeed due to an increase in *luc* transcript levels, the transcripts were quantified (Fig. 5). The levels of transcripts were normalized with respect to an endogenous transcript from the host cell (tRNA$_1^{Gly}$, levels of which do not change drastically following virus infection; Sharma *et al.*, 1997), as well as to the amounts of transfected DNA (Fig. 5*d*). A clear increase in the level of *luc* transcripts was detected in all the samples that were transfected with plasmids containing the enhancer *in cis*, compared with those lacking it or the minimal promoter construct.

Transcription status of the enhancer fragment

In order to check whether the region containing the enhancer element (nt 127170–127463 on the BmNPV genome) was itself transcriptionally active, RNase protection assays were carried out using antisense riboprobes corresponding to the individual ORFs contained within this

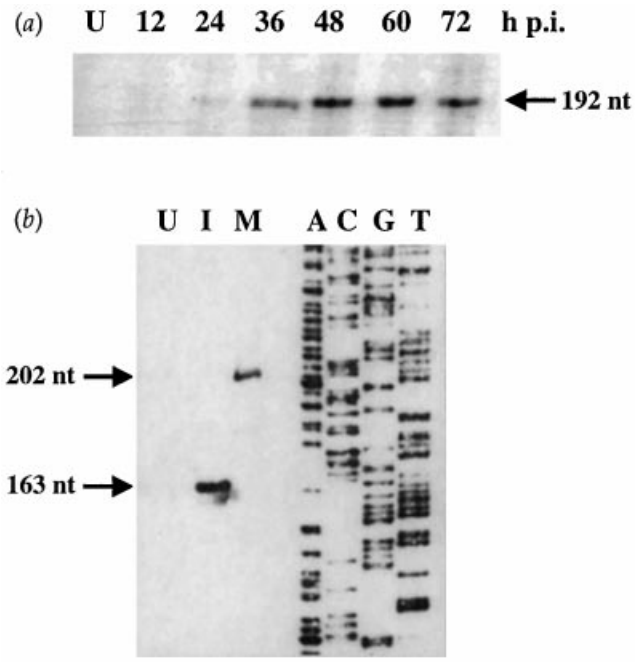

Fig. 6. RNase protection analysis of pBmMinpolhR3$^{+/-}$. The transcriptional status of the enhancer-containing region *in vivo* was determined by RNase protection analysis. (*a*) Transcription of ORF453. Plasmid pBmMinpolhR3^{+} was linearized with *Mlu*I and transcribed *in vitro* in the presence of T3 RNA polymerase to generate an antisense riboprobe of 330 nt encompassing 145 bp of ORF453, starting from the ATG, and the rest of the upstream region. The labelled antisense riboprobe was hybridized at 50 °C in 50% formamide for 12 h with 20 µg total RNA isolated from uninfected (U) or BmNPV-infected cells at 12, 24, 36, 48, 60 and 72 h p.i. and digested with RNase A and RNase T1. The protected fragment of 192 nt corresponding to the ORF453 transcript is shown. (*b*) Transcriptional status of ORF327. RNA corresponding to ORF327 was detected by a labelled antisense riboprobe generated from pBmMinpolhR3^{-}. The ^{32}P-labelled probe was synthesized *in vitro* using T7 RNA polymerase from the linearized plasmid using [α-^{32}P]UTP as the radioactive label and processed as described for (*a*). The 163 nt protected fragment detected from infected cells (I) at 60 h p.i. is shown. Lane U, RNA from uninfected cells. A sequencing ladder and an *in vitro*-synthesized 202 nt *luc* RNA (lane M) were used to size the protected fragment.

fragment. A 192 nt protected fragment corresponding to the ORF453 transcript, which followed a delayed expression pattern (the transcript first appearing at 36 h p.i. and peaking between 48 and 60 h p.i.), was clearly seen (Fig. 6*a*). The distal late transcription start site TAAG, mapping at −40 nt from the +1 ATG of ORF327, is common to *lef-2* and ORF327 (Sriram & Gopinathan, 1998). A 163 nt protected fragment of ORF327 was seen when the corresponding antisense probe was used (Fig. 6*b*). Thus, both ORF453 and ORF327 were transcribed in the course of virus infection.

Activation from heterologous promoters

Since enhancer sequences are known to bring about activation of heterologous promoters as well, the effect of this enhance-like element on another very late gene promoter (*p10* promoter) was analysed. The enhancer-containing *Mlu*I fragment was cloned upstream of the AcMNPV *p10* promoter in the transfer vector pUW1, carrying the reporter gene *luc* (pUW1M). Expression from this construct showed only a 2-fold stimulation of luciferase activity (Fig. 3, right panel). However, expression from the *p10* promoter itself (in the parental plasmid pUW1) was very high, almost 20 times that observed in the case of pBmpolh.

Discussion

We have identified a region in the upstream flanking sequences of *polh* of BmNPV, encompassing ORF453 and ORF327, that enhances *polh* promoter-based expression. The 293 bp fragment was found to satisfy the following criteria for an enhancer element: (i) it augmented transgene expression directed by the *polh* promoter in a position- and orientation-independent manner, (ii) no stimulation of the *polh* promoter was observed *in trans*, (iii) increased expression of the reporter gene, concomitant with increased transcription levels, was observed and (iv) expression was stimulated without promoter specificity. The enhancement of transcriptional activity was only 10-fold, compared with the 1000-fold stimulation observed from the *hr*-based enhancers. The enhancer-harbouring fragments alone could not restore the minimal promoter activity back to that of the parental plasmid pBmpolh, suggesting that even the remaining regions upstream of *polh* are important in fostering maximal levels of transcription. No repeat elements characteristic of enhancer elements could be identified in the enhancer region analysed here. However, two AP1 sites (TGACTCG), separated from each other by 40 nt, were identified in this region. Subfragments of the enhancer region containing one or both the AP1 motifs, when placed upstream of the minimal promoter, did not stimulate expression from the *polh* minimal promoter. Although the 101 bp subfragment, harbouring two AP1 sites, formed a specific complex with nuclear extracts from infected BmN cells (but not with uninfected cell extracts) in gel mobility-shift assays, the complex was not chased out with an excess of AP1 sequences (data not shown). Taken together, these results imply that the AP1 sites may not be involved in the enhancer function. The minimal enhancer element constituted the N-terminal coding regions of ORF453 and ORF327, which belong to the class of uncharacterized genes in all known baculoviruses. The region encompassing the enhancer is actively transcribed (Fig. 6*a*, *b*) from both strands, a feature so far not reported in other conventional enhancers. The significance, if any, of the transcription of this enhancer region is not clear. We therefore define this newly identified region as an enhancer-like element, to distinguish it from conventional enhancers.

The enhancer fragment identified here has been tested with two very late viral promoters, *polh* and *p10*. Although these promoters are transcribed exclusively by the virus-encoded polymerase induced late in the infection (Guarino *et al.*, 1998), host factors have also been reported to play a role in late gene

transcription (Burma *et al.*, 1994; Mukherjee *et al.*, 1995). However, since extracts from uninfected cells failed to yield a complex with a segment of the enhancer fragment (data not shown), the involvement of early or late virus factors serving as activators through this enhancer element is more likely. For the highest levels of expression from the *polh* promoter containing the entire 4·0 kb 5′ upstream region of the promoter (as in pVL1392), the optimal placement of the foreign gene is at +35 nt with respect to the +1 ATG (Luckow & Summers, 1989). In vector pBm030, the reporter gene is located at −3 nt in relation to the *polh* ATG, and the expression levels are much lower (10–20% of the former; Sriram *et al.*, 1997). Even though the *p10* promoter in vector pUW1 contains only 300 nt upstream of the +1 ATG, it still provides an expression level of 60% compared with the optimized *polh* promoter-based vector, with its full-length 5′ upstream region. Activity from the minimal AcMNPV *polh* promoter (harbouring up to 550 nt of 5′ upstream sequences) was much lower (20%) compared with *p10*-based expression (Sriram *et al.*, 1997; Sriram & Gopinathan, 1998; Palhan & Gopinathan, 2000).

Although both *p10* and *polh* are transcribed as very late genes by the same virus-encoded polymerase, the requirements for *cis* elements appear to be somewhat different. Earlier studies on expression from these two promoters suggested competition for one or more components (Chaabihi *et al.*, 1993; van Oers *et al.*, 1992). Deletion of the *p10* promoter boosted *polh* gene expression, presumably through the liberation of one or more components involved in *polh* transcription. In contrast, no transcriptional enhancement from the *p10* promoter was seen when the *polh* gene was deleted, suggesting the requirement for *p10*-specific factors that are limiting. The activation of *p10* occurs a few hours earlier than that of *polh* and the maximum expression levels attained are much lower (Roelvink *et al.*, 1992). Furthermore, except for the consensus core sequence TAAG motif at the transcriptional initiation site, the leader sequences of *polh* and *p10* share only limited nucleotide sequence similarity, implying different requirements for transcription factors. This may be the basis for differential regulation of the two very late viral promoters, despite being transcribed by the same virus RNA polymerase. We extend this hypothesis to the case of the enhancer-like region identified here, since the extent of stimulation observed for the two promoters was not the same. Although the 2·3 kb *polh* 5′ upstream regions of BmNPV and AcMNPV differed significantly (absence of ORF603 and presence of *bro-e* in BmNPV), the 293 bp region was present in both baculoviruses and showed more than 95% identity. However, the 293 bp region was located at different distances, 0·9–1·2 kb upstream of the +1 ATG of *polh* in BmNPV and 1·6–1·9 kb upstream in AcMNPV. In both viruses, the *hr*1 sequences are located about 4 kb upstream of the polyhedrin start site. The very high level of expression from the *polh* promoter achieved *in vivo* is likely to be the combined effect of the enhancer-like element(s) and the *hr* sequences.

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References

Ahrens, C. H. & Rohrmann, G. F. (1995). Replication of *Orgyia pseudotsugata* baculovirus DNA: *lef-2* and *ie-1* are essential and *ie-2*, *p34*, and *Op-iap* are stimulatory genes. *Virology* **212**, 650–662.

Ahrens, C. H., Russell, R. L., Funk, C. J., Evans, J. T., Harwood, S. H. & Rohrmann, G. F. (1997). The sequence of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. *Virology* **229**, 381–399.

Ayres, M. D., Howard, S. C., Kuzio, J., Lopez-Ferber, M. & Possee, R. D. (1994). The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* **202**, 586–605.

Blissard, G. W. & Rohrmann, G. F. (1990). Baculovirus diversity and molecular biology. *Annual Review of Entomology* **35**, 127–155.

Burma, S., Mukherjee, B., Jain, A., Habib, S. & Hasnain, S. E. (1994). An unusual 30-kDa protein binding to the polyhedrin gene promoter of *Autographa californica* nuclear polyhedrosis virus. *Journal of Biological Chemistry* **269**, 2750–2757.

Chaabihi, H., Ogliastro, M. H., Martin, M., Giraud, C., Devauchelle, G. & Cerutti, M. (1993). Competition between baculovirus polyhedrin and p10 gene expression during infection of insect cells. *Journal of Virology* **67**, 2664–2671.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156–159.

Gearing, K. L. & Possee, R. D. (1990). Functional analysis of a 603 nucleotide open reading frame upstream of the polyhedrin gene of *Autographa californica* nuclear polyhedrosis virus. *Journal of General Virology* **71**, 251–262.

Gomi, S., Majima, K. & Maeda, S. (1999). Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. *Journal of General Virology* **80**, 1323–1337.

Guarino, L. A. & Dong, W. (1994). Functional dissection of the *Autographa californica* nuclear polyhedrosis virus enhancer element *hr5*. *Virology* **200**, 328–335.

Guarino, L. A. & Summers, M. D. (1986). Interspersed homologous DNA of *Autographa californica* nuclear polyhedrosis virus enhances delayed-early gene expression. *Journal of Virology* **60**, 215–223.

Guarino, L. A., Xu, B., Jin, J. & Dong, W. (1998). A virus-encoded RNA polymerase purified from baculovirus-infected cells. *Journal of Virology* **72**, 7985–7991.

Habib, S., Pandey, S., Chatterji, U., Burma, S., Ahmad, R., Jain, A. & Hasnain, S. E. (1996). Bifunctionality of the AcMNPV homologous region sequence (*hr*1): enhancer and *ori* functions have different sequence requirements. *DNA and Cell Biology* **15**, 737–747.

Hayakawa, T., Rohrmann, G. F. & Hashimoto, Y. (2000). Patterns of genome organization and content in lepidopteran baculoviruses. *Virology* **278**, 1–12.

Kang, W., Suzuki, M., Zemskov, E., Okano, K. & Maeda, S. (1999). Characterization of baculovirus repeated open reading frames (*bro*) in *Bombyx mori* nucleopolyhedrovirus. *Journal of Virology* **73**, 10339–10345.

Keddie, B. A., Aponte, G. W. & Volkman, L. E. (1989). The pathway of infection of *Autographa californica* nuclear polyhedrosis virus in an insect host. *Science* **243**, 1728–1730.

Kuzio, J., Pearson, M. N., Harwood, S. H., Funk, C. J., Evans, J. T., Slavicek, J. M. & Rohrmann, G. F. (1999). Sequence and analysis of the genome of a baculovirus pathogenic for *Lymantria dispar*. *Virology* **253**, 17–34.

Leisy, D. J. & Rohrmann, G. F. (1993). Characterization of the replication of plasmids containing *hr* sequences in baculovirus-infected *Spodoptera frugiperda* cells. *Virology* **196**, 722–730.

Lu, M., Farrell, P. J., Johnson, R. & Iatrou, K. (1997). A baculovirus (*Bombyx mori* nuclear polyhedrosis virus) repeat element functions as a powerful constitutive enhancer in transfected insect cells. *Journal of Biological Chemistry* **272**, 30724–30728.

Luckow, V. A. & Summers, M. D. (1989). High level expression of nonfused foreign genes with *Autographa californica* nuclear polyhedrosis virus expression vectors. *Virology* **170**, 31–39.

Maeda, S. (1989). Gene transfer vectors of a baculovirus, *Bombyx mori* nuclear polyhedrosis virus, and their use for expression of foreign genes in insect cells. *In Invertebrate Cell System Applications*, pp. 167–182. Edited by J. Mitsuhashi. Boca Raton, FL: CRC Press.

Merrington, C. L., Kitts, P. A., King, L. A. & Possee, R. D. (1996). An *Autographa californica* nucleopolyhedrovirus *lef-2* mutant: consequences for DNA replication and very late gene expression. *Virology* **217**, 338–348.

Mukherjee, B., Burma, S. & Hasnain, S. E. (1995). The 30-kDa protein binding to the 'initiator' of the baculovirus polyhedrin promoter also binds specifically to the coding strand. *Journal of Biological Chemistry* **270**, 4405–4411.

Ooi, B. G., Rankin, C. & Miller, L. K. (1989). Downstream sequences augment transcription from the essential initiation site of a baculovirus polyhedrin gene. *Journal of Molecular Biology* **210**, 721–736.

O'Reilly, D. R., Miller, L. K. & Luckow, V. E. (1992). *Baculovirus Expression Vectors: A Laboratory Manual*. New York: W. H. Freeman.

Palhan, V. B. & Gopinathan, K. P. (2000). The *p10* gene of *Bombyx mori* nucleopolyhedrosis virus encodes a 7·5 kDa protein and is hyper-transcribed from a TAAG motif. *Journal of Genetics* **79**, 33–40.

Palhan, V. B., Sumathy, S. & Gopinathan, K. P. (1995). Baculovirus mediated high-level expression of luciferase in silkworm cells and larvae. *Biotechniques* **19**, 97–104.

Pearson, M., Bjornson, R., Pearson, G. & Rohrmann, G. F. (1992). The *Autographa californica* baculovirus genome: evidence for multiple replication origins. *Science* **257**, 1382–1384.

Possee, R. D. & Howard, S. C. (1987). Analysis of the polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus. *Nucleic Acids Research* **15**, 10233–10248.

Rankin, C., Ooi, B. G. & Miller, L. K. (1988). Eight base pairs encompassing the transcriptional start point are the major determinant for baculovirus polyhedrin gene expression. *Gene* **70**, 39–49.

Roelvink, P. W., van Meer, M. M. M., de Kort, C. A. D., Possee, R. D., Hammock, B. D. & Vlak, J. M. (1992). Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and p10 genes. *Journal of General Virology* **73**, 1481–1489.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sharma, S., Sriram, S., Patwardhan, L. & Gopinathan, K. P. (1997). Expression of individual members of a $tRNA_1^{Gly}$ multigene family *in vivo* follows the same pattern as *in vitro*. *Gene* **194**, 257–266.

Sriram, S. (1998). *Bombyx mori nucleopolyhedrosis virus: expression from very late promoters*. PhD thesis, Indian Institute of Science, Bangalore.

Sriram, S. & Gopinathan, K. P. (1998). The potential role of a late gene expression factor, *lef2*, from *Bombyx mori* nuclear polyhedrosis virus in very late gene transcription and DNA replication. *Virology* **251**, 108–122.

Sriram, S., Palhan, V. B. & Gopinathan, K. P. (1997). Heterologous promoter recognition leading to high-level expression of cloned foreign genes in *Bombyx mori* cell lines and larvae. *Gene* **190**, 181–189.

van Oers, M. M., Malarme, D., Jore, J. M. P. & Vlak, J. M. (1992). Expression of the *Autographa californica* nuclear polyhedrosis virus p10 gene: effect of polyhedrin gene expression. *Archives of Virology* **123**, 1–11.

Zemskov, E. A., Kang, W. & Maeda, S. (2000). Evidence for nucleic acid binding ability and nucleosome association of *Bombyx mori* nucleopolyhedrovirus BRO proteins. *Journal of Virology* **74**, 6784–6789.

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