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Characterization of late gene expression factors *lef*-9 and *lef*-8 from *Bombyx mori* nucleopolyhedrovirus

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Late gene expression factors, LEF-4, LEF-8, LEF-9 and P47 constitute the primary components of the *Autographa californica* multinucleocapsid polyhedrovirus (AcMNPV)-encoded RNA polymerase, which initiates transcription from late and very late promoters. Here, characterization of *lef*-9 and *lef*-8, which encode their corresponding counterparts, from *Bombyx mori* NPV is reported. Transcription of *lef*-9 initiated at two independent sites: from a GCACT sequence located at −38 nt and a CTCTT sequence located at −50 nt, with respect to the +1 ATG of the open reading frame. The 3′ end of the transcript was mapped to a site 17 nt downstream of a canonical polyadenylation signal located 7 nt downstream of the first of the two tandem translational termination codons. Maximum synthesis of LEF-9 was seen from 36 h post-infection (p.i.). The transcription of *lef*-8 initiated early in infection from a GTGCAAT sequence that differed in the corresponding region from its AcMNPV counterpart (GCGCAGT), with consequent elimination of the consensus early transcription start site motif (underlined). Peak levels of *lef*-8 transcripts were attained by 24 h p.i. Immunocopurification analyses suggested that there was an association between LEF-8 and LEF-9 *in vivo*.

Introduction

The strong promoters of the viral very late genes *polyhedrin* (*polh*) and *p10* are most frequently employed in baculovirus-based expression vector systems to drive heterologous gene expression (reviewed by Blissard & Rohrmann, 1990; King & Possee, 1992). Next to *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV) in popularity amongst baculoviruses for recombinant protein expression is *Bombyx mori* NPV (BmNPV) (Palhan *et al.*, 1995; Sehgal & Gopinathan, 1998; Acharya & Gopinathan, 2001). Although the complete genomic sequence of BmNPV is available (Gomi *et al.*, 1999), much less is known about the basic molecular biology and gene expression patterns of this natural pathogen of the mulberry silkworm, *B. mori*. Gene expression in NPVs follows a regulated cascade, controlled predominantly at the transcriptional level (Friesen & Miller, 1985). The key regulatory event in this cascade is the transition from early to late gene expression, which employs a polymerase switching mechanism. Early viral gene transcription, brought about by host RNA polymerase II (sensitive to α-amanitin), is independent of the virus-encoded protein products or viral DNA replication. In contrast, the late and very late genes are transcribed by an α-amanitin-resistant RNA polymerase, which is synthesized during infection (Grula *et al.*, 1981; Fuchs *et al.*, 1983), and their promoters harbour a consensus signature motif, TAAG.

In AcMNPV, transient expression with overlapping clones representing the entire genome identified a set of genes called late gene expression factors (*lef*s), essential for expression from late and very late promoters (Lu & Miller, 1994, 1995; Todd *et al.*, 1995; Li *et al.*, 1999). Their homologues have been identified in various baculoviruses whose genomes have been completely sequenced (Ayres *et al.*, 1994; Gomi *et al.*, 1999; Kuzio *et al.*, 1999; Ahrens *et al.*, 1997). Of these, the products encoded by *lef*s 4, 5, 6, 8, 9, 10, 11, 12, *38K* and *p47* are believed to regulate late gene expression at the level of transcription. In addition, very late gene expression factor 1 (encoded by *vlf*-1) was required for transcription from very late gene promoters (McLachlin & Miller, 1994; Yang & Miller, 1998, 1999). Deletion analysis of all the *lef*s in BmNPV has demonstrated that except for four *lef*s, *ie-2*, *39K*, *lef-7* and *p35*, the others were essential for virus propagation *in vitro* (Gomi *et al.*, 1997). However, molecular characterization of only a few

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of the BmNPV *lef*s has been reported so far (Sriram & Gopinathan, 1998; Mikhailov, 2000).

The differences in promoter structure as well as the nature of polymerase capable of initiating transcription from late and very late promoters supported the notion of a virus-encoded/modified polymerase in baculoviruses. Besides being insensitive to α-amanitin, the viral polymerase also differed from the host polymerase in cofactor requirements (Grula *et al*., 1981; Fuchs *et al*., 1983). Beniya *et al*. (1996) purified the AcMNPV RNA polymerase that could accurately initiate transcription from late (*p6.9*) and very late (*polh*) promoters. The minimal constituents of the polymerase were subsequently identified to be LEF-4, LEF-8, LEF-9 and P47 (Guarino *et al*., 1998a). LEF-8 shows homology to the second largest β-subunit of prokaryotic DNA-directed RNA polymerase, harbouring the conserved $GXKX_4HGQ/NKG$ motif (Passarelli *et al*., 1994), whereas LEF-9 is homologous to the largest β′-subunit in harbouring the conserved NADFDGD sequence motif (Lu & Miller, 1994). LEF-4 from AcMNPV has been characterized as the mRNA capping enzyme (Guarino *et al*., 1998b; Jin *et al*., 1998). The BmNPV counterpart of LEF-4 also carries out all the enzymatic functions related to mRNA capping activity (S. Sehrawat & K. P. Gopinathan, unpublished data). The function or presence of any recognizable motifs in P47 is not reported but the protein has been localized to the nucleus of infected cells (Carstens *et al*., 1993, 1994). Recently, 3′ polyadenylation activity has also been demonstrated to be an inherent property of the viral polymerase (Jin & Guarino, 2000). The transcriptional regulation of most of the *lef*s, however, has not been analysed in detail.

In this study, we describe the cloning and characterization of *lef*-9 and *lef*-8 from BmNPV. Detailed transcriptional analyses revealed that *lef*-9 transcripts initiated at multiple sites different from the consensus baculovirus early transcription start site motif CAGT and terminated downstream to a canonical polyadenylation sequence. Using antibodies raised against the bacterially expressed LEF-9, the synthesis of the protein in infected cells was also monitored. Preliminary studies on the transcriptional mapping of *lef*-8 as well as the possible interaction between LEF-8 and LEF-9 in BmNPV-infected BmN cells have also been carried out.

Methods

■ **Cell line and virus.** The *B. mori*-derived cell line, BmN, was propagated at 27 °C in TC-100 medium supplemented with 10% foetal bovine serum (Gibco BRL). For all temporal expression profile analyses as well as transient transfection studies, the BmNPV BGL strain (a local isolate of BmNPV) (Palhan & Gopinathan, 1996) was used. Virus stocks were maintained and titrated according to standard protocols (O'Reilly *et al*., 1992). Transfections were carried out with 2–5 μg DNA (CsCl purified) per 1×10^6 cells using lipofectin (Palhan *et al*., 1995). After 1 h, transfection medium was removed and the cells were infected in complete medium with BmNPV at an m.o.i. of 10. After 48 h at 27 °C, the cells were harvested and washed with PBS.

■ **Generation of plasmid constructs.** The synthetic oligonucleotides used as primers for PCR, cloning, sequencing and primer extensions are shown in Figs 3 and 5(c). Plasmid construct pRPU9, harbouring the N-terminal region and immediate 5′ upstream sequences of *lef*-9 used in RNase protection analysis of *lef*-9 transcripts, was constructed by PCR amplification of a 210 bp fragment from BmNPV DNA using primers P3 and P4 and cloning at the *Eco*RV site of pBS-SK$^+$. For expression of *lef*-9 ORF in *Escherichia coli*, *lef*-9 was amplified from viral genomic DNA using primers P1 and P2 and *Pfu* DNA polymerase and inserted into plasmid pET32a (harbouring a His-tag as well as a thioredoxin fusion sequence) as a 1·5 kb *Bam*HI–*Eco*RI fragment (clone designated pTrxALef-9). To study the transcription profile of *lef*-8, a genomic fragment encoding the C-terminal region of the gene and the downstream sequences encompassing the *etm* open reading frame (ORF) was amplified as a 566 bp fragment using primers P7 and P8 and cloned in pBS-KS$^+$ (clone designated pCL8). For expression as a FLAG epitope-tagged protein in insect cells, the *lef*-8 clone was generated in three steps. The 5′ region (480 bp) of *lef*-8, starting from + 1 ATG of the ORF, was amplified using primers P9 and P10 and cloned as a *Bam*HI–*Xho*I fragment in pBS-SK$^+$. The remaining part of *lef*-8 was derived from the clone pBmXJ (harbouring the 4·1 kb *Xho*I J fragment of BmNPV genomic DNA in pBS-SK$^+$) as a 3·0 kb *Xho*I–*Eco*RI fragment. The 3·5 kb full-length *lef*-8 gene together with downstream sequences was mobilized as a *Bam*HI–*Eco*RI fragment under the control of the BmNPV *p10* promoter harbouring the FLAG epitope tag (clone designated pFLef-8). The BmNPV *p10* promoter harbouring the FLAG tag was constructed in our laboratory (V. B. Palhan & K. P. Gopinathan, unpublished results) by inserting a 57-mer synthetic oligonucleotide (5′ CATTTTATTTAACTATCATCTCATGGACTAC-AAAGACG-ACGACGACAAAGGATCCCG 3′) encoding the FLAG peptide (N–Asp–Tyr–Lys–Asp–Asp–Asp–Asp–Lys–C) downstream of the BmNPV *p10* promoter.

■ **RNase protection, primer extension, Northern blotting and 3′ RACE.** Total RNA from uninfected- and BmNPV-infected BmN cells at various times post-infection (p.i.) was isolated by the guanidinium–isothiocyanate method (Chomczynski & Sacchi, 1987) and treated with RNase-free DNase I (10 U per 1×10^7 cells). Expression profiles of *lef*-9 and *lef*-8 were analysed by RNase protection assays using the appropriate complementary RNA probes. Antisense riboprobes of high specific activity were generated *in vitro* from the cloned BmNPV genomic DNA fragments in plasmid pBS-SK$^+$ using either T3 or T7 RNA polymerase in the presence of radiolabelled [α-^{32}P]UTP. Total RNA (20–40 μg) was coprecipitated with the corresponding antisense riboprobes ($1{\cdot}5 \times 10^5$ c.p.m.) in the presence of 200 mM NaCl and 20 μg carrier DNA using 2·5 vols ethanol. Following hybridization at 50 °C for 16 h in the presence of 50% formamide, the RNase digestion mixture containing RNase A (2 U) and RNase T1 (1 U) was added and, after 1 h at 37 °C, the samples were precipitated in the presence of 10 μg yeast tRNA (added as carrier) and 2·5 vols ethanol. The RNase-protected samples were analysed by electrophoresis on 6% acrylamide gels containing 7 M urea and visualized by autoradiography.

Transcription start sites for *lef*-8 and *lef*-9 were determined by primer extension analysis. Total RNA (20–40 μg) was annealed to 5 pmol of the appropriate primer and reverse transcription was performed using Superscript II Reverse Transcriptase (Gibco BRL) in the presence of [α-^{32}P]dATP (10 μCi; 3000 Ci/mmol) and 100 pmol each of dCTP, dGTP and dTTP for 5 min at 42 °C. This was followed by extension reactions for 5 min in the presence of vast excesses of all four dNTPs (200 μM each). The reaction was terminated using 80% formamide gel-loading dye containing 200 μM EDTA. The primer-extended products were analysed on 6% acrylamide gels containing 7 M urea together with the

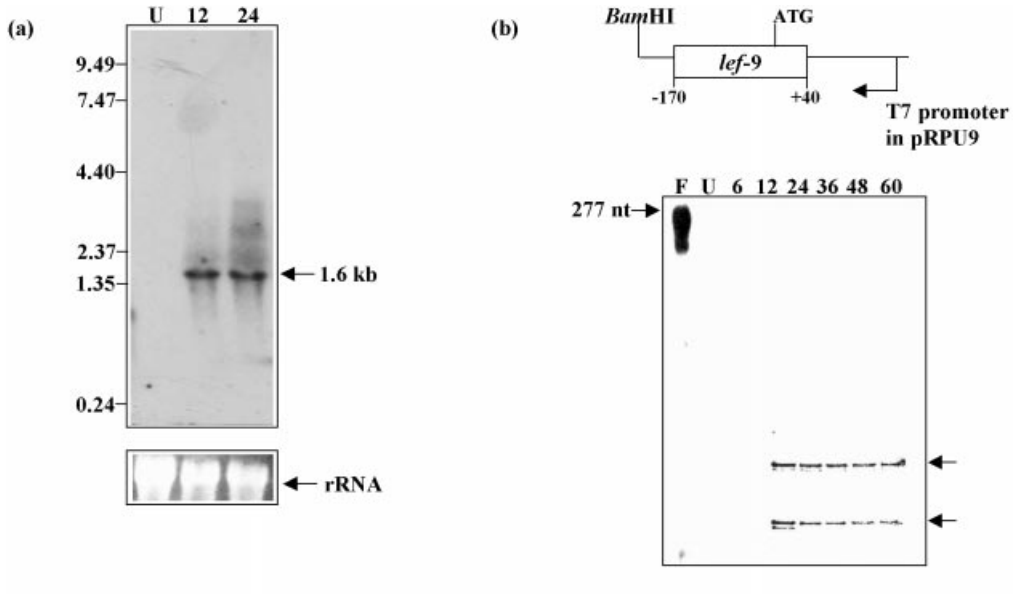

Fig. 1. Temporal expression profiles of BmNPV *lef*-9 RNA. (a) Northern blot analysis of *lef*-9 RNA. Total RNA (40 μg) isolated from uninfected (lane U) and BmNPV-infected BmN cells (m.o.i. of 10) at 12 and 24 h p.i. (lanes 12 and 24) was resolved on a 1·2% MOPS–formaldehyde agarose gel, transferred onto a nylon membrane and hybridized to a radiolabelled *lef*-9 probe. The signal corresponding to *lef*-9 is marked by an arrow. To indicate the amounts of RNA loaded, an ethidium bromide staining of the same gel showing the 28S rRNA levels is shown in the lower panel. (b) Temporal expression profiles of *lef*-9 analysed by RNase protection assays. The strategy for generating the antisense probe is shown schematically on the top panel. The 277 nt probe comprising −170 to +40 nt of *lef*-9 (and the rest from vector sequences) was hybridized with 20 μg total RNA from uninfected (lane U) or BmNPV-infected BmN cells at 6, 12, 24, 36, 48 and 60 h p.i. and subjected to RNase protection analysis. The RNase-protected fragments corresponding to the multiple transcripts were detected by autoradiography (marked by arrows).

appropriate DNA sequencing ladders for sizing. Bands were detected by autoradiography.

Northern blotting was carried out as described by Sambrook *et al.* (1989). Total RNA (40 μg) isolated from control as well as BmNPV-infected BmN cells was separated on a 1·2% MOPS–formaldehyde agarose gel and transferred onto a N^+ nylon membrane (Amersham Pharmacia). The blot was probed using a radiolabelled full-length *lef*-9-specific probe, which was generated by random priming. Following hybridization (16 h at 42 °C in the presence of 50% formamide), the blots were washed at a final stringency of 65 °C in 0·1 × SSC and 0·1% SDS and autoradiographed.

The 3′ end of the *lef*-9 transcript was mapped precisely by RACE. Reverse transcriptions were performed with total RNA using the 3′ RACE adapter primer, 5′ GGCCACGCGTCGACTAGTAC$(T)_{17}$ 3′, and Superscript II Reverse Transcriptase at 42 °C for 1 h. Following RNase H treatment at 30 °C for 30 min, PCR amplification was carried out with one-tenth of the volume of the above reaction and *Vent* DNA polymerase using the *lef*-9 N-terminal primer P1 (encompassing the +1 ATG) and the 3′ RACE anchor primer, 5′ GGCCACGCGTCGACTAGTAC 3′. This was followed by two rounds of amplification with the *lef*-9 RACE forward primer P5 (50 nt upstream of the ORF stop codon) and the 3′ RACE anchor primer. The amplified product was cloned in pBS-SK^+ at the *Eco*RV site and sequenced with the *lef*-9 forward primer P5 to precisely map the transcription termination site.

■ **Polyclonal antisera and Western blotting.** Rabbit polyclonal antiserum was raised against LEF-9 expressed as a thioredoxin fusion protein with a C-terminal His-tag (clone pTrxALef-9) in *E. coli* strain BL-21. The bacterially expressed protein was purified through affinity chromatography in an Ni–NTA agarose column. The purified protein (800 μg) was injected into a rabbit in the presence of Freund's complete adjuvant followed by three rounds of boosters, each with 500 μg of the purified protein (in Freund's incomplete adjuvant) administered at an interval of 10 days; the rabbit serum was checked for the presence of antibodies to LEF-9 by Western blot.

To analyse the temporal synthesis of LEF-9, uninfected as well as BmNPV-infected BmN cells (1×10^5) were suspended in SDS gel loading buffer [50 mM Tris (pH 6·8), 2% SDS, 1% β-mercaptoethanol and 10% glycerol] and analysed on an 8% polyacrylamide gel containing 0·1% SDS. Following electrophoresis, the proteins were electrophoretically transferred onto a PVDF membrane at 1·0 mA/cm^2 for 1 h. The membrane was blocked with 3% gelatin overnight and probed with a 1:1000 dilution of the anti-LEF-9 antiserum followed by incubation with secondary anti-rabbit goat antibody conjugated to horseradish peroxidase. After extensive washing, the blot was developed using the ECL + Plus Western Blot Detection kit (Amersham Pharmacia).

To study the interaction between LEF-8 and LEF-9 *in vivo*, the FLAG-tagged construct pFLef-8 was transfected into BmN cells, (2·5 μg DNA per 1×10^6 cells) in serum-free medium. After 8 h, the cells were infected with BmNPV (m.o.i. of 10) in TC-100 complete medium. At 48 h p.i., the cells were harvested, washed with PBS and lysed with 1% NP-40 in 10 mM Tris (pH 7·9), 10 mM NaCl, 1·5 mM $MgCl_2$, 5 mM DTT and 10% glycerol. The nuclei were pelleted by centrifugation at 3000 r.p.m. for 10 min. The nuclear proteins were extracted in extraction buffer [50 mM Tris (pH 7·4), 500 mM NaCl, 5 mM DTT and 1 mM EDTA] containing 1% Triton-X-100. The sample was then diluted to 150 mM NaCl in TBS and bound to an anti-FLAG M2 affinity gel, previously equilibrated with TBS. After 1 h of binding at 4 °C, the matrix was

washed with TBS and bound proteins were eluted using 0·1 M glycine or competitive elution with the FLAG peptide (25 nmol). This fraction was analysed by Western blotting using the FLAG- or LEF-9-specific antibodies and the ECL + Plus Western Blot Detection kit.

Results

Transcriptional analysis of *lef*-9

Initial studies on the transcription pattern of *lef*-9 in BmNPV-infected BmN cells by Northern blots revealed a single transcript of ~ 1·6 kb at 12 as well as 24 h p.i. (Fig. 1a). The temporal expression profile of *lef*-9 was therefore analysed through the entire course of virus infection by the more sensitive RNase protection assay (Fig. 1b). Transcript signals due to *lef*-9 were undetected at 6 h p.i. but evident at 12 h p.i. and their levels stayed fairly constant until 60 h p.i. Two distinct transcripts, differing by about 12 nt, were clearly discernable. To further confirm the presence of the two transcripts and to map their 5′ ends, primer extension analysis was carried out (Fig. 2a). The smaller transcripts (76 and 78 nt long) initiated from the G and A residues of the sequence GCACT located 22 nt downstream of a TATAT sequence in this generally AT-rich region. The other 90 nt transcript mapped to the first C of a CTCTT sequence, located 10 nt downstream of the same TATAT sequence. Evidently, the transcription start site sequences were different from the consensus baculovirus early (CAGT) and late (TAAG) transcription start site motifs.

The transcription termination site of *lef*-9 was mapped by 3′ RACE of the transcript by sequencing the RACE product after cloning. A canonical polyadenylation signal, AATAAA, was located 7 nt downstream of the first of the two tandem translation termination codons of the ORF and a poly(A) stretch of 53 residues was added 17 nt downstream of this motif (Fig. 2b). Thus, the total length of the transcript as determined from the precise mapping of the 5′ and 3′ ends matched well with the size of the transcript (1·6 kb) seen in Northern blot. The results from transcript analysis of *lef*-9 with respect to the structure of the gene are presented in Fig. 3.

Immunodetection of LEF-9 in BmN cells

Synthesis of LEF-9 in BmNPV-infected BmN cells was analysed by Western blot using the polyclonal antibodies

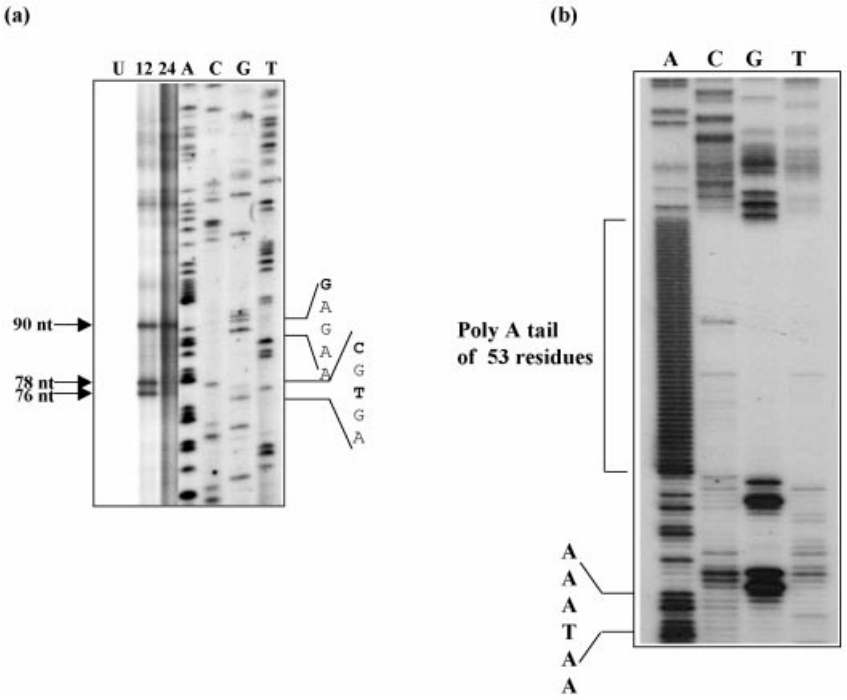

Fig. 2. Mapping of *lef*-9 transcription start and termination sites. (a) Transcription start site mapping by primer extension. Total RNA (20 μg) from uninfected (lane U) or BmNPV-infected BmN cells (m.o.i. of 10) at 12 and 24 h p.i. (lanes 12 and 24) was hybridized to primer P4 located at +40 nt from the +1 ATG of *lef*-9 (for sequence and locations of primers, see Fig. 3) and subjected to primer-extension analysis. The transcription start sites were mapped based on the sequencing ladder generated for *lef*-9 [clone pRPU9, shown in Fig. 1(b) and using the same primer (lanes A, C, G and T)]. Transcription start sites are indicated by arrows and the corresponding sequences of the complementary strands are provided next to the sequencing ladder and are marked in bold. (b) Mapping the 3′ terminus of *lef*-9 transcript by RACE. Total RNA (10 μg) from BmNPV-infected BmN cells at 12 h p.i. was reverse transcribed in the presence of 3′ RACE adapter primer. For details of assay, see Methods. The PCR amplified product (~ 150 bp) was cloned into pBS-SK$^+$ at the *Eco*RV site and sequenced using primer P5. The consensus polyadenylation signal AATAAA and the poly(A) stretch are marked.

Fig. 3. Nucleotide sequence of *lef*-9 region of BmNPV. Nucleotide sequences encompassing the *lef*-9 region (nt 44221–45960 on the BmNPV genome) are shown. The predicted amino acid sequences, in parts, of LEF-9 ORF are indicated below the nucleotide sequence in capital letters (numbers marked on the right margin). The two tandem translation termination signals of ORF (marked with asterisks) and the locations of various primers P1 to P5 used in PCR amplification or primer extensions are indicated. Primers P1 and P2 used in PCR amplification to clone and express the ORF in bacterial vectors had *Eco*RI linkers at their 5′ ends. The TATA box-like sequence (TATAT), the transcription start sequences CTCTT and GCACT, as well as the consensus polyadenylation signal (AATAAA) are indicated in bold. The site of poly(A) addition is marked by an arrowhead.

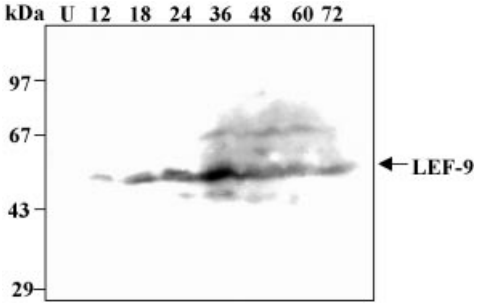

Fig. 4. Synthesis of LEF-9 in BmNPV-infected cells. Temporal profile of LEF-9 synthesis in BmNPV-infected BmN cells. Total cell lysates from 1×10^5 uninfected (lane U) or BmNPV-infected BmN cells (lanes 12, 18, 24, 36, 48, 60, 72 h p.i.) were resolved on an 8% polyacrylamide gel containing 0·1% SDS and transferred onto a PVDF membrane. The blot was blocked by treatment with 0·3% gelatin overnight and probed using polyclonal anti-LEF-9 antibodies, followed by binding to goat anti-rabbit IgG conjugated to horseradish peroxidase. The signals due to the substrate reaction were visualized using the ECL+Plus Western Blot Detection kit.

raised against the bacterially expressed purified protein (Fig. 4). A 52 kDa band, corresponding to LEF-9, appeared from 12 h p.i. and maximal levels were seen at 36 h p.i. in BmNPV-infected cells; levels of expression remained high until later times in infection. The protein showed slight anomalous mobility (52 kDa) as compared to the predicted size of the protein (56·4 kDa).

Transcriptional analysis of *lef*-8

The *lef*-8 gene of BmNPV was isolated from an *Xho*I library of BmNPV genomic DNA based on the sequence information available (GenBank accession no. L33180) (Gomi *et al*., 1999). The gene was located in two contiguous *Xho*I fragments of 4·1 and 2·2 kb, corresponding to the J and L fragments (nt 33691–37756 and 37756–39999, respectively) on the BmNPV genome. The temporal transcription profile of *lef*-8 was analysed by RNase protection and primer extension assays (Fig. 5). The antisense probe used for RNase protection spanned 467 nt of the *lef*-8 C terminus, including the translational stop codon, and 99 nt downstream running into the next partially deleted (*etm*) sequences (Fig. 5a, top panel). A protected fragment of ~560 nt corresponding to the *lef*-8 transcript was detected from 12 to 72 h p.i. (the first and the last time-points tested) (Fig. 5a). The highest levels of *lef*-8 transcript expression were seen at 24 h p.i. Even though the entire *etl* sequences as well as the 5′ 150 bp of *etm*, located immediately downstream of *lef*-8 in AcMNPV, are deleted in BmNPV (Fig. 5c), the size of the RNase-protected fragment indicated that the transcript originating in the *lef*-8 region ran well into the residual *etm* sequences. The transcripts of *p*10 (a very late gene used as a control) were detected at 24 h p.i., increased by 36 h p.i. and stayed at very high levels until 72 h p.i. (Fig. 5a, lower panel).

The 5′ upstream sequences of BmNPV *lef*-8 also did not reveal the presence of any known early and late transcription start site motifs. To precisely map the 5′ end of the *lef*-8 transcript, primer extension analysis was carried out using a primer located at 250 bp downstream of the +1 ATG of the ORF (primer P6). A strong signal due to the primer-extended product of 269 nt was detected at both 12 and 24 h p.i. (Fig. 5b). The transcription start site corresponded to the first G of a GTGCAAT sequence, as deduced from the sequencing ladder of the region generated using the same primer. This G residue was located 19 nt upstream from the +1 ATG of the ORF and the sequence differed from the corresponding region in AcMNPV at two positions, marked by underlined text, of the G<u>C</u>GCA<u>G</u>T. These base changes resulted in the loss of the consensus early transcription start site motif CAGT in BmNPV. Although the region immediately upstream of the start site was AT-rich, no TATA box-like sequences were present and, therefore, the BmNPV *lef*-8 transcription start site did not map to any known consensus early or late motifs.

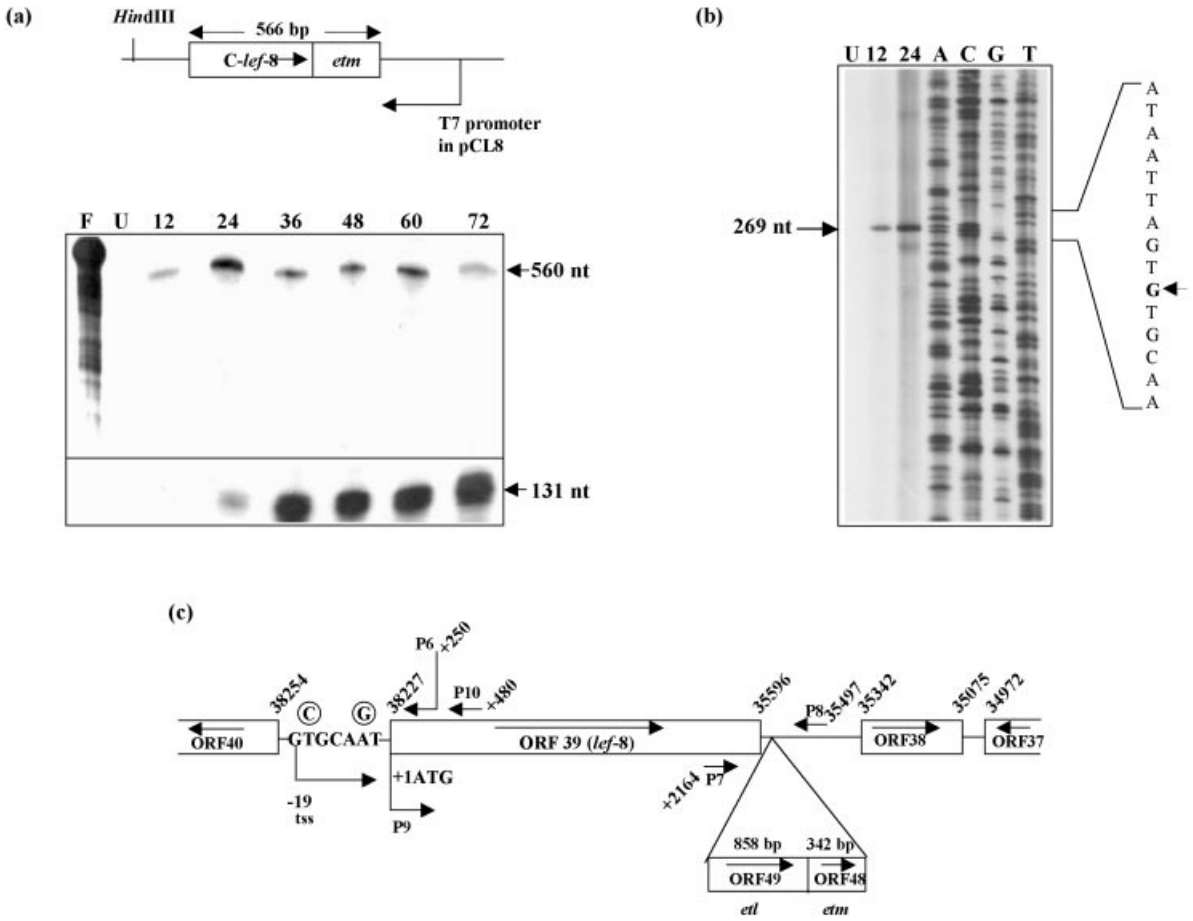

Fig. 5. Transcription profiles and 5′ end mapping of BmNPV *lef*-8. (a) Temporal expression profiles of *lef*-8 were analysed by RNase protection analysis. The strategy for generating the antisense *lef*-8 probe is shown schematically (top panel). The 650 nt radiolabelled probe, comprising 467 nt of the C-terminal region of *lef*-8 encompassing the translational stop together with 99 nt corresponding to the downstream *etm* sequences, and 84 nt of the vector sequences, was hybridized to 20 μg total RNA from uninfected (lane U) or BmNPV-infected cells (at 12, 24, 36, 48, 60 and 72h p.i.). The samples were then subjected to RNase protection analysis. The approximate size of the transcript (560 nt) was deduced from the DNA molecular size markers and the free probe. The transcript levels of the very late gene *p10* in the same RNA samples were also determined using the corresponding antisense probe, to serve as a control (lower panel, transcript size 131 nt). (b) Primer extension of *lef*-8 transcripts. Total RNA (30 μg) isolated from either uninfected (lane U) or BmNPV-infected (m.o.i. of 10) BmN cells at 12 and 24 h p.i. (lanes 12 and 24) were used for primer extension analysis in presence of the primer P6 (250 nt downstream of the +1 ATG). The transcription start site was mapped based on the sequencing ladder (lanes A, C, G and T) generated from *lef*-8 (cloned L fragment from the *Xho*I library of BmNPV genomic DNA) using the same primer. The transcription start site is marked and the corresponding sequence (complementary strand) is indicated next to the sequencing ladder. (c) Schematic diagram of the *lef*-8 (ORF39) region of BmNPV showing the major differences with the same region of AcMNPV. The different ORFs, their location on the BmNPV genome and the positions of the various primers used for PCR amplification and primer extension are marked. The orientation of *lef*-8 on BmNPV genome is opposite to that of *polh*. The coordinates for the primers P6, P7, P9 and P10 as well as the transcription start site are marked with respect to the +1 ATG of the *lef*-8 ORF. The primers used were: P6, 5′ cgtcgatatcgtccgtttc 3′: for transcription start site mapping by primer extension; P7, 5′ taaaaatatgtggcattcac 3′, and P8, 5′ aggtgtcgtcttcgaca 3′: forward and reverse primers for PCR amplification and cloning the region encoding the C-terminal domain used in RNase protection; P9, 5′ ggggtaccgaattcggatccatgacggacgtagttc 3′, and P10, 5′ cgtacacgtctcgagcaaac 3′: forward and reverse primers for PCR amplification and cloning of the N-terminal encoding region.

Transcriptional mapping of the *lef*-8 region is presented schematically in Fig. 5(c). The major differences between AcMNPV and BmNPV in this region [e.g. complete deletion of AcMNPV ORF49 (*pcna*) and partial deletion of ORF48 (*etm*) from the BmNPV genome], the base changes in the transcription start site regions (circled) and the primers used for different purposes are all marked.

Interaction between BmNPV LEF-8 and LEF-9

Since both LEF-8 and LEF-9 have been demonstrated to be the subunits of the baculoviral RNA polymerase in the prototype AcMNPV (Guarino *et al.*, 1998), the possible interaction between the counterparts of these two subunits from BmNPV was examined. In preliminary studies using the

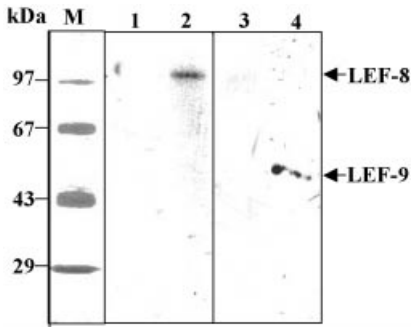

Fig. 6. Interaction between LEF-8 and LEF-9. BmN cells, transfected with pFLef-8 (a construct harbouring FLAG-tagged *lef*-8 under the BmNPV *p10* promoter) and infected with BmNPV (m.o.i. of 10), were harvested at 48 h p.i. and processed by binding to the immunoaffinity matrix. Bound proteins were eluted using 0·1 M glycine or by competitive elution with 25 nmol FLAG peptide, resolved by electrophoresis on an 8% acrylamide gel containing 0·1% SDS and Western blotted. Blots were probed using mouse anti-FLAG (lane 2) or rabbit anti-LEF-9 (lane 4) antibodies and the antibody reactions were detected using the ECL+Plus Western Blot Detection kit. An appropriate control in which only the control plasmid (pBS-SK$^+$) but not pFLef-8 was transfected prior to BmNPV infection was included and the nuclear proteins were processed identically (lanes 1 and 3). Lanes: M, molecular size markers; 1 and 2, probed with anti-FLAG antibodies; 3 and 4, probe with anti-LEF-9 antibodies.

yeast two-hybrid system, we could not demonstrate any interaction between them. Therefore, a coimmunoprecipitation approach was attempted in which *lef*-8 was expressed as a FLAG-tagged protein, under the control of the strong viral very late *p*10 promoter, by transfection of pFlef-8 into BmN cells followed by infection with BmNPV. The transiently expressed FLAG-tagged LEF-8, together with interacting viral proteins (resulting from BmNPV infection), was purified on an anti-FLAG M2-affinity matrix. Western blotting of the eluates from the affinity matrix with monoclonal anti-FLAG and polyclonal anti-LEF-9 antibodies detected bands of $\sim$ 102 and 52 kDa, respectively (Fig. 6, lanes 2 and 4, respectively). The signals corresponding to the FLAG-tagged LEF-8 and the viral LEF-9 proteins suggested that the two proteins either interacted directly or constituted part of a complex *in vivo*. In control samples (where the cells were not transfected with the FLAG-tagged *lef*-8 construct) when the nuclear proteins were passed through FLAG-specific antibody affinity column, no immunoreactive bands were detected with anti-FLAG or anti-LEF-9 antibodies (Fig. 6, lanes 1 and 3, respectively).

Discussion

Transcription from the late and very late promoters in AcMNPV is executed by a virally encoded polymerase comprising equimolar amounts of viral late gene expression factors LEF-8, LEF-9, LEF-4 and P47 (Guarino *et al*., 1998). This minimal unit was sufficient to initiate transcription from the viral late and very late promoters *in vitro*. We have analysed the expression patterns of two of the genes, *lef*-8 and *lef*-9, encoding the corresponding subunits in BmNPV. Both *lef*-8 and *lef*-9 were transcribed from 12 h p.i. in BmNPV-infected BmN cells. Since BmNPV growth rates are slower compared to AcMNPV and DNA replication generally commences between 12 and 18 h p.i. (compared to 6–12 h p.i. in AcMNPV), these transcription profiles can be taken as that of early transcripts. The transcription start sites in both instances did not conform to the known consensus early or late transcription start motifs, CAGT or TAAG. However, it is known that nearly 30–50% of the mapped early transcripts do not initiate from CAGT motifs.

LEF-8 and LEF-9 being constituents of the viral polymerase required for transcription of late and very late promoters should, ideally, be expressed early in infection and, presumably, by the host RNA polymerase. It is likely that their transcription also depends on the early virus transactivators or host factors. The transcription start site for *lef*-8 is located at -19 nt from the $+1$ ATG of the ORF, mapping to the first G residue of the sequence GTGCAAT. In AcMNPV, the transcription start site of *lef*-8 is not mapped but the sequence in the corresponding region is GCGCAGT (differing from BmNPV at two bases and thus harbouring the early transcription start site motif CAGT). The sequences downstream of *lef*-8 also show major differences between the two viruses. This region in AcMNPV (29·0–30·1 map units) encompasses three ORFs, encoded by *etl*, *etm* and *ets*, located immediately downstream of *lef*-8 (Ayres *et al*., 1994). The largest of these, *etl*, encodes a 28 kDa polypeptide expressed early in infection that shows homology to the eukaryotic DNA polymerase δ processivity factor, PCNA. The disruption of *etl* had no effect on virus viability (Crawford & Miller, 1988). The other two early ORFs, encoding ETM and ETS, have not been assigned any function. In BmNPV, this region harbours a 1·1 kb deletion resulting in the complete loss of *etl* and 150 nt from the 5′ region of *etm* (Gomi *et al*., 1999). The analysis of BmNPV *lef*-8 transcripts by RNase protection revealed that the transcripts extended to the remaining *etm* region. The first potential polyadenylation signal (AATAAA) after the two tandem termination codons of LEF-8 was 130 nt downstream and 132 nt upstream of the $+1$ ATG of the adjoining *ets* ORF. Our efforts to map the 3′ end of *lef*-8 transcript were not successful due to the low abundance of the transcript and the limitations in electrophoretic resolution of RNase-protected fragments, which were larger when other primer combinations available to us were used. The large size of the *lef*-8 transcript and its possible instability were also responsible for the extensive degradation observed in Northern blots (data not shown).

The 5′ end mapping of *lef*-9 transcripts revealed the presence of multiple transcription start sites. One of these transcription start sites, GCACT, differed from the consensus early motif CAGT by 1 nt, but the other, CTCTT, did not fall into any of the known consensus motifs. The sequences reported here were similar to those in AcMNPV (Guarino *et al*., 1998a). The shorter transcripts initiating from the GCACT

sequence were detected only at 12 h p.i., whereas the more distal transcription start site at CTCTT was preferentially utilized at later time-points. The significance of these multiple initiation sites is not clear at present. The precise sites of transcription termination and poly(A) addition of *lef*-9 transcripts mapped here demonstrated the utilization of the consensus polyadenylation signal, located 7 nt downstream of the first of the two tandem translation termination codons of the LEF-9 ORF. This consensus motif was followed by an immediate downstream U-rich sequence implicated in transcript processing in most AcMNPV mRNAs as well as other eukaryotic transcripts (Westwood *et al*., 1993; MacLauchlan *et al*., 1985; McDevitt *et al*., 1986).

Although LEF-8 and LEF-9 harbour the conserved RNA polymerase subunit motifs, so far no independent functions have been identified. Being constituents of the virally encoded polymerase, a possible interaction or association between these subunits was predictable. Our attempts to demonstrate a direct interaction between LEF-8 and LEF-9 of BmNPV by yeast two-hybrid analysis did not show any interaction between them (data not presented). However, in the preliminary studies reported here, an association of these two proteins *in vivo* could be demonstrated by immunocoprecipitation exploiting a FLAG-tagged *lef*-8 construct. Our results suggest that, *in vivo*, a subcomplex of LEF-8 and LEF-9 may be weak or the association with the rest of the polymerase subunits is essential to form a stable complex.

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References

Acharya, A. & Gopinathan, K. P. (2001). Identification of an enhancer-like element in the polyhedrin gene upstream region of *Bombyx mori* nucleopolyhedrovirus. *Journal of General Virology* **82**, 2811–2819.

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.

Beniya, H., Funk, C. J., Rohrmann, G. F. & Weaver, R. F. (1996). Purification of a virus-induced RNA polymerase from *Autographa californica* nucleopolyhedro virus-infected *Spodoptera frugiperda* cells that accurately initiates late and very late transcription *in vitro*. *Virology* **216**, 12–19.

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

Carstens, E. B., Lu, A. L. & Chan, H. L. B. (1993). Sequence, transcriptional mapping, and overexpression of p47, a baculovirus gene regulating late gene expression. *Journal of Virology* **67**, 2513–2520.

Carstens, E. B., Chan, H., Yu, H., Williams, G. V. & Casselman, R. (1994). Genetic analyses of temperature-sensitive mutations in baculovirus late expression factors. *Virology* **204**, 323–337.

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

Crawford, A. M. & Miller, L. K. (1988). Characterization of an early gene accelerating expression of late genes of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *Journal of Virology* **62**, 2773–2781.

Friesen, P. D. & Miller, L. K. (1985). Temporal regulation of baculoviral RNA: overlapping early and late transcripts. *Journal of Virology* **54**, 392–400.

Fuchs, L. Y., Woods, M. S. & Weaver, R. F. (1983). Viral transcription during AcNPV infection: a novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. *Journal of Virology* **48**, 641–646.

Gomi, S., Zhou, C. E., Yih, W., Majima, K. & Maeda, S. (1997). Deletion analysis of four of eighteen late gene expression factor gene homologues of the baculovirus, BmNPV. *Virology* **230**, 35–47.

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

Grula, M. A., Buller, P. L. & Weaver, R. F. (1981). α-amanitin-resistant viral RNA synthesis in nuclei isolated from nuclear polyhedrosis virus infected *Heliothios zea* larvae and *Spodoptera frugiperda* cells. *Journal of Virology* **38**, 916–921.

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

Guarino, L. A., Jin, J. & Dong, W. (1998b). Guanylyltransferase activity of the LEF-4 subunit of baculovirus RNA polymerase. *Journal of Virology* **72**, 10003–10010.

Jin, J. & Guarino, L. A. (2000). 3′-end formation of baculovirus late RNAs. *Journal of Virology* **74**, 8930–8937.

Jin, J., Dong, W. & Guarino, L. A. (1998). The LEF-4 subunit of baculovirus RNA polymerase has RNA 5′-triphosphatase and ATPase activities. *Journal of Virology* **72**, 10011–10019.

King, L. A. & Possee, R. D. (1992). *The Baculovirus Expression System: a Laboratory Guide*. London: Chapman & Hall.

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.

Li, L., Harwood, S. H. & Rohrmann, G. F. (1999). Identification of additional genes that influence baculovirus late gene expression. *Virology* **255**, 9–19.

Lu, A. & Miller, L. K. (1994). Identification of three late gene expression factors within the 33·8- to 43·4-map-unit region of *Autographa californica* nuclear polyhedrosis virus. *Journal of Virology* **68**, 6710–6718.

Lu, A. & Miller, L. K. (1995). The roles of eighteen baculovirus late gene expression factor genes in transcription and DNA replication. *Journal of Virology* **69**, 975–982.

McDevitt, M. A., Hart, R. P., Wong, W. W. & Nevins, J. R. (1986). Sequences capable of restoring poly(A) site function define two distinct downstream elements. *EMBO Journal* **5**, 2907–2913.

McLachlin, J. R. & Miller, L. K. (1994). Identification and characterization of *vlf*-1, a baculovirus gene involved in very late gene expression. *Journal of Virology* **68**, 7746–7756.

McLauchlan, J., Gaffnay, D., Whitton, J. L. & Clements, J. B. (1985).

The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNA 3′ termini. *Nucleic Acids Research* **13**, 1347–1368.

Mikhailov, V. S. (2000). Helix-destabilizing properties of the baculovirus single-stranded DNA-binding protein (LEF-3). *Virology* **270**, 180–189.

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. (1996). Characterization of a local isolate of *Bombyx mori* nuclear polyhedrosis virus. *Current Science* (*Bangalore*) **70**, 147–153.

Palhan, V. B., Sumathy, S. & Gopinathan, K. P. (1995). Baculovirus mediated high-level expression of luciferase in silkworm cells and larvae. *Biotechniques* **19**, 97–105.

Passarelli, A. L., Todd, J. W. & Miller, L. K. (1994). A baculovirus gene involved in late gene expression predicts a large polypeptide with a conserved motif of RNA polymerases. *Journal of Virology* **68**, 4673–4678.

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

Sehgal, D. & Gopinathan, K. P. (1998). Recombinant *Bombyx mori* nucleopolyhedrovirus harboring green fluorescent protein. *Biotechniques* **25**, 997–1004.

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

Todd, J. W., Passarelli, A. L. & Miller, L. K. (1995). Eighteen baculovirus genes, including *lef*-11, *p35*, *39K*, and *p47*, support late gene expression. *Journal of Virology* **69**, 968–974.

Westwood, J. A., Jones, I. M. & Bishop, D. H. L. (1993). Analyses of alternative poly(A) signals for use in baculovirus expression vectors. *Virology* **195**, 90–99.

Yang, S. & Miller, L. K. (1998). Control of baculovirus polyhedrin gene expression by very late factor 1. *Virology* **248**, 131–138.

Yang, S. & Miller, L. K. (1999). Activation of baculovirus very late promoters by interaction with very late factor 1. *Journal of Virology* **73**, 3404–3409.

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