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 _GCACGT_ 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 z-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 z-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 (lefS), 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 lefS 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 lefS in BmNPV has demonstrated that except for four lefS, _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 lefs 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 (puli) 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 GXXX_HQQ/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., 1999b; 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 lefs, 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 (CaCl₂ purified) per 1 x 10⁶ 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 EcoRV site of pBS-SK+ (BamH I-EcoRI fragment (clone designated pTrxALEf-9). To study the transcription profile of lef-9, a genomic fragment encoding the C-terminal region of the gene and the downstream sequences encompassing the 3′ open reading frame (ORF) was amplified as a 560 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 BamH I–Xhol fragment in pBS-SK+. The remaining part of lef-8 was derived from the clone pBmXJ (harbouring the 41 kbp Xhol I fragment of BmNPV genomic DNA in pBS-SK+) as a 30 kbp Xhol–EcoRI fragment. The 3.5 kbp full-length lef-8 gene together with downstream sequences was mobilized as a BamH I–EcoRI fragment under the control of the BmNPV p10 promoter harbouring the FLAG epitope tag (clone designated pFeL-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′-CATTTTTATTTAACTATCATCTCATGGACTAC-AAAAGCAG-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 x 10⁶ 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 [α-3²P]UTP. Total RNA (20–40 μg) was coprecipitated with the corresponding antisense riboprobes (1.5 x 10⁶ c.p.m.) in the presence of 200 mM NaCl and 20 μg carrier DNA using 2.5 μl 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 RNA (added as carrier) and 2-5 μl 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-3²P]dATP (10 μCi; 3000 Ci/mmole) 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
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 % MOPS-formaldehyde agarose gel, transferred onto a 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 x 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' GCCCACCGCTCGACTAGTAC(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' GCCCACCGCTCGACTAGTAC 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 EcoRV 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 x 106) 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 10 mA/cm² 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 pLeF-8 was transfected into BmN cells, (25 µg DNA per 1 x 106 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), 150 mM NaCl, 1 mM MgCl₂, 5 mM DTT and 10 % glycerol. The nuclei were pelleted by centrifugation at 3500 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
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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 GCAG located 22 nt downstream of a TATAT sequence. 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...
The temporal transcription profile of lef-8 gene was isolated from an XhoI 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 XhoI 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 p10 (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 GGGCAGT. 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 XhoI 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′ cgtgatctgtcggctc 3′: for transcription start site mapping by primer extension; P7, 5′ taaaaatatggcctgcag 3′, and P8, 5′ aggggatctggctc 3′: forward and reverse primers for PCR amplification and cloning the region encoding the C-terminal domain used in RNase protection; P9, 5′ gggtaccggcaatctggctgccgagcagcagcgc 3′, and P10, 5′ cgctagctgcgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgct
immunoreactive bands were detected with anti-FLAG or anti-LEF-9 antibodies; 3 and 4, probe with anti-LEF-9 antibodies. 

Fig. 6. Interaction between LEF-8 and LEF-9. BmN cells, transfected with pLeF-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 immunofinity 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. Lanes 2 and 4, probed with anti-FLAG antibodies; 3 and 4, probe with anti-LEF-9 antibodies.

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 vivo. 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 GCAGCGT (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 (290–301 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 ETS 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 5′ 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, GCAGT, differed from the consensus early motif CAGT by 1 nt, but the other, CTCCCT, 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 GCAGT
sequence were detected only at 12 h p.i., whereas the more distal transcription start site at CTCCTT 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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