Expression and localization of LEF-11 in Autographa californica nucleopolyhedrovirus-infected Sf9 cells

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The Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) lef-11 gene was found previously to be necessary to support optimal levels of transient expression from an AcMNPV late promoter. The lef-11 gene is unusual in that it overlaps both upstream (orf38) and downstream (pp31) genes. In this study, the expression and cellular localization of LEF-11 were examined. The lef-11 transcripts were detected from 4 to 36 h post-infection (p.i.). The 1.5 kb lef-11 mRNA initiates 196 nt upstream of the lef-11 translation initiation codon, within the upstream orf38 gene. This relatively long 5′ upstream region encodes a potential small upstream open reading frame (ORF) of 58 amino acids that overlaps the lef-11 ORF. The 3′ end of the lef-11 mRNA was mapped as co-terminal with mRNAs from the downstream pp31 gene. Using affinity purified anti-LEF-11 antibodies, levels of LEF-11 expression were found to be maximal between approximately 8 and 24 h p.i., although LEF-11 could be detected as late as 72 h p.i. Using immunofluorescence microscopy, it was determined that LEF-11 localized to dense regions of infected cell nuclei, consistent with its role as a possible late transcription factor.

The Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) has a large DNA genome (133-9 kb) and transcription of viral genes occurs in a cascade fashion, resulting in early, late and very late phases of transcription (Ayres et al., 1994; Friesen, 1997; Lu & Miller, 1997). Early gene expression and viral DNA replication are required for late and very late gene expression. While early genes are transcribed by the host RNA polymerase II, late and very late genes are transcribed by an α-amanitin-resistant viral RNA polymerase (Fuchs et al., 1983; Glocker et al., 1993; Grula et al., 1981; Huh & Weaver, 1990). Using a transient late expression assay system (Passarelli & Miller, 1993), 19 lef genes were found to be necessary to support optimal levels of transient expression from an AcMNPV late promoter (Li et al., 1999; Lu & Miller, 1995; Rapp et al., 1998; Todd et al., 1995). Of the 19 lef genes identified in this manner, 10 appear to be involved in DNA replication and approximately 11 are thought to be involved more directly in late transcription. Biochemical analysis of a purified late RNA polymerase complex identified AcMNPV proteins LEF-4, -8, -9 and P47 as major components of the late RNA polymerase (Gross & Shuman, 1998; Guarino et al., 1993). Of the 19 lef genes, such as lef-11, resulted in a reduction of, but not a complete loss of, reporter activity when compared with a similar assay containing a full complement of lef genes (Lu & Miller, 1995). The low level of transcription activity that appeared to remain suggests that proteins such as LEF-11 may represent accessory transcription factors important for regulation of transcription by the late RNA polymerase, but perhaps not necessary for basal levels of transcription.

Although the lef-11 open reading frame (ORF) was identified by sequence analysis and its expression was implied by complementation experiments in transient late transcription assays, the lef-11 gene transcripts were not previously mapped nor was the presence of the protein documented or localized within infected cells. The lef-11 ORF is somewhat unusual in that it is small and overlaps both the orf38 and the pp31 ORFs. Thus, if expressed as a discrete unit of transcription, the lef-11 promoter may be located within the upstream orf38 gene. In the current study, we examined lef-11 transcription and expression during the infection cycle. To study the expression of the lef-11 gene in the context of an AcMNPV infection, we used 5′ and 3′ RACE analysis as well as Northern blot analysis to examine RNAs transcribed from the region containing the lef-11 gene.

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Fig. 1. Mapping the 5′ and 3′ ends of *lef-11* mRNAs by 5′ and 3′ RACE and Northern blot analysis. (A) Organization of the *lef-11* gene region of AcMNPV. Locations and orientations of the *orf38*, *lef-11* and *pp31* ORFs (open boxes), the small overlapping ORF (grey box) and mapped mRNAs (dashed lines) are shown. Locations of the gene-specific primers (arrows) used for 5′ RACE analysis (*lef-11-GSP1*, 5′-GTTTGGCTCTGTAAACCTTTGAAACAACCC-3′, and *lef-11-GSP2*, 5′-GTGCTCTAGATGATATATTTTTTAAATGCC-3′) and 3′ RACE analysis (*lef11–3′ RACE*, 5′-GAACCCGGATCCCGTCTGCGCGCACATGTTGGAC-3′, and *lef11/pp31–3′ RACE*, 5′-CCGGATCCCGGGTTGTTTCAAAGGTTTACAAGAAG-3′) are indicated. The cRNA probe (Northern probe) used for Northern blots is indicated below the *lef-11* ORF. (B) PCR products generated from 5′ RACE analysis of the *lef-11* region are shown on the agarose gel and labelled according to the relative location of the transcription start site and the major ORF immediately downstream (*lef-11* or *pp31*). Sizes of a DNA standard marker (M) are indicated in kbp on the left of the gel. The sequence alignments on the left show the nucleotide sequence of the 5′ RACE product aligned with the AcMNPV genomic DNA sequence from the *lef-11* and the *pp31* gene regions. For RACE analysis, PCR products were cloned into the SalI–XbaI site of pBluescript and sequenced. (C) The agarose gel shows PCR products generated by 3′ RACE of the *lef-11* gene using primers specific for the detection of the *lef-11* transcripts alone (lane a, solid arrowhead) or for both the *lef-11* and the *pp31* transcripts (lane b, open arrowhead). Smaller non-specific products found in the lower portion of the gel (bracket) are also indicated. The sequence alignment on the left shows the location of the 3′ end mapped from both 3′ RACE products (*lef-11* and *lef-11 + pp31*). The position of a consensus poly(A) signal (AATAAAA) is underlined. (D) Northern blot analysis of *lef-11* gene expression. A strand-specific cRNA probe (Northern probe, as seen in A) was hybridized to a Northern blot of RNAs isolated from AcMNPV-infected Sf9 cells (m.o.i. = 10) at various times p.i. Two major mRNA species (1-5 and 1-1 kb) are indicated and these RNAs correspond to RNAs mapped by 5′ and 3′ RACE analysis (dashed lines, as seen in A). Numbers above the lanes indicate times (h) p.i. when RNAs were isolated (Sf9, mock-infected cells). Relative locations of RNA size markers (kb) are indicated on the left. Each lane on the Northern blot represents 5 µg of total RNA. RNAs were electrophoresed on 1-2% formaldehyde agarose gels, blotted onto magnacharge nylon transfer membrane (MSI) and...
To map the 5′ ends of RNAs transcribed from the lef-11 ORF, two gene-specific primers were used for nested PCR to amplify and clone the 5′ ends of the lef-11 transcripts by 5′ RACE (Fig. 1A, B). Using total RNA isolated from AcMNPV-infected Sf9 cells at 12 h post-infection (p.i.) and lef-11-specific primers, two products were detected after 5′ RACE analysis: an abundant product of approximately 200 bp and a less abundant product of approximately 520 bp. Cloning and sequencing of the 200 bp 5′ RACE product revealed the 5′ end of an early transcript that initiates within the lef-11 ORF and corresponds to a previously mapped early transcript encoding the downstream pp31 ORF. The pp31 ORF overlaps the 3′ end of the lef-11 ORF by 4 bp (Figs 1A and 2). Sequence analysis of the 520 bp 5′ RACE product revealed a single transcription initiation site located 196 nt upstream of the lef-11 ORF and 25 nt downstream of a putative TATA box (Figs 1A, B and 2) at nt 30597 on the AcMNPV C6 genome sequence (Ayres et al., 1994). This transcription start site is within the orf38 ORF and, thus, the lef-11 promoter is probably within the orf38 ORF. Interestingly, a Met codon is found on the relatively long (196 nt) 5′ leader sequence of this lef-11 RNA and this Met codon is 139 nt upstream of the lef-11 ORF and in the same frame as the orf38 ORF (Fig. 2, underlined sequence). Translation initiation from this upstream Met codon would result in the translation of a 174 nt ORF encoding 58 amino acids. This small upstream ORF does not terminate prior to the initiation of the lef-11 ORF, but overlaps it by 35 nt and is in a different reading frame. We resequenced this region and confirmed that the nucleotide sequence of this region of the AcMNPV genome (GenBank accession no. L22858.1 GI:510708) was correct. If initiation occurs at the upstream Met codon, it is possible that this might result in a substantial downregulation of LEF-11 translation. Minicistrons (very small ORFs) in the 5′ leader sequences of several baculovirus genes have been reported previously (Blissard & Rohrmann, 1989; Guarino & Smith, 1990; Lu & Carstens, 1992) and translation of a minicistron was shown to downregulate translation of the downstream ORF on gpe4 late mRNAs (Chang & Blissard, 1997). Because previous studies using transient late transcription assays demonstrated that the lef-11 ORF was functional (Rapp et al., 1998), it is likely that the lef-11 ORF (nt 30401–30063) is discretely translated. Initiation at the lef-11 Met codon could occur via a ‘leaky-scanning’ mechanism in which the upstream Met codon is not used, or is inefficiently used, as a translation initiator.

The 3′ ends of the lef-11 transcripts were determined by 3′ RACE analysis using total RNA extracted at 12 h p.i. Because the 5′ ends of the pp31 transcripts overlap the lef-11 ORF, we designed two primers to identify the 3′ end of the lef-11 transcripts. One primer was specific for the lef-11 transcripts, while the second detected both the pp31 and the lef-11 transcripts (Fig. 1A, lef-11–3′ RACE and lef11/pp31–3′ RACE). Sequencing the 3′ RACE products generated from both primers revealed that both major 3′ RACE products mapped to 3′ ends at the same site, indicating that both the pp31 and the lef-11 transcripts share a common 3′ end (Figs 1A, C and 2). The 3′ ends of both transcripts were mapped to a position that corresponds to nt 29222, located 20 nt downstream of a consensus poly(A) signal (Figs 1C and 2). Sequencing of several smaller products generated by 3′ RACE (Fig. 1C, lane b, bracket) revealed only non-AcMNPV sequences; we identified no 3′ ends unique to the lef-11 transcripts. Taken together, data from mapping the 5′ and 3′ ends predicted a lef-11 transcript of 1376 nt, excluding the poly(A) tail, consistent with a lef-11 RNA detected by Northern blot analysis using a lef-11 gene probe (see below). Similarly, these data also identified a pp31 transcript of 976 nt.

To examine the temporal nature of lef-11 transcription, we used Northern blot analysis. A 278 nt strand-specific cRNA probe was labelled with [α-32P]ATP (Fig. 1A) and hybridized to Northern blots containing total RNA isolated from AcMNPV-infected Sf9 cells at various times p.i. (Fig. 1D). Four RNAs were detected by hybridization with the lef-11 probe (Fig. 1D). Two RNAs of 1·5 and 1·1 kb correspond to mapped transcripts from the lef-11 and the pp31 genes, respectively. Two additional bands observed on these blots appear to be artefacts, as they were not identified by either 5′ or 3′ RACE analysis of this region. Attempts to use a shorter cRNA probe specific for the lef-11 gene alone were unsuccessful, probably due to limitations on the size of these probes and the relatively low abundance of the lef-11 transcripts. Because the lef-11 ORF overlaps the pp31 ORF and the transcription start site of the pp31 gene is within the lef-11 ORF, the cRNA probe used in this study (Fig. 1A, Northern Probe) detected both the lef-11 and the pp31 transcripts. Mapping the 5′ and 3′ ends of the lef-11 RNA by RACE analysis identified a 1·376 kb RNA species, consistent with the identification of a 1·5 kb RNA by Northern blot analysis using a lef-11 cRNA probe (Fig. 1D). The 1·5 kb lef-11 mRNA was detected as early as 4 h p.i. and was most abundant between 12 and 24 h p.i. The steady-state levels of the 1·5 kb lef-11 mRNA appear to be much lower than those of the 1·1 kb pp31 mRNA, since the 278 nt probe was completely homologous to the 1·5 kb mRNA, yet the intensity of the 1·5 kb band was dramatically lower than that of the 1·1 kb...
Fig. 2. The nucleotide sequences of the orf38, lef-11, pp31 loci are shown, together with the locations of the mapped transcription initiation sites (arrows) and the 3' end (arrowhead). The amino acid sequence encoded by an ORF immediately upstream and overlapping the lef-11 ORF is underlined and the lef-11 amino acid sequence is shown in bold. Nucleotide sequences corresponding to a poly(A) signal (AATAAA) are also shown in bold. Nucleotide sequence numbers are indicated on the left. The overlaps between the orf38, small upstream ORF, lef-11 and pp31 ORFs are indicated.

lef-11 ORF

pp31 ORF

orf38

band. Only 115 nt (nt 30084–30198) of the 278 nt probe were homologous to the 1 kb pp31 mRNA (Fig. 1A, D). The 1 kb pp31 RNA was the most abundant transcript detected. The size and quantity of this transcript are consistent with 5' and 3' end mapping of the pp31 gene in this and previous studies (Guarino & Smith, 1990, 1992). The 1 kb pp31 mRNA was detected from 4 to 72 h p.i. and was most abundant between 24 and 36 h p.i. (Fig. 1D). Detection of the pp31 transcripts during the late phase was expected, since a pp31 late promoter characterized previously is located immediately upstream of the pp31 gene early transcription start site (Guarino & Smith, 1992). The lef-11 ORF encodes a predicted protein of 112 aa with a molecular mass of approximately 13–1 kDa. The predicted LEF-11 protein contains a single putative zinc finger motif (Cys–X6–Cys–X5–Phe–X18–Leu–X18–His–X18–His) near the N terminus and a basic-charged region near the C terminus. To examine LEF-11 expression in infected cells, an anti-LEF-11 antiserum against a six His-tagged LEF-11 protein expressed in Escherichia coli was generated in rabbits. Anti-LEF-11 antibodies were affinity purified and used for Western blot analysis of extracts from AcMNPV-infected Sf9 cells. Using anti-LEF-11 antibodies, a protein with a molecular mass of approximately 16 kDa was detected in AcMNPV-infected Sf9 cells from 4 to 72 h p.i. (Fig. 3A). Maximal levels of LEF-11 were detected at 12 h p.i., which coincides with times at which maximal levels of the lef-11 RNAs were detected (Fig. 1D). Although lef-11 mRNA levels declined and were not readily detectable after 36 h p.i., LEF-11 was detected up to 72 h p.i., suggesting that LEF-11 may be relatively stable during the late phase of infection. Although the LEF-11 band appears to be lower in intensity at 36 h p.i., it is not clear how this can be explained. The low intensity of the lef-11 band at 36 h p.i. may represent either low levels of LEF-11 or perhaps an experimental artefact. To examine the cellular localization of LEF-11, we also used affinity purified anti-LEF-11 antibodies for immunofluorescence microscopy of AcMNPV-infected Sf9 cells (Fig. 3B). Anti-LEF-11 antibodies were prepared by preadsorption with Sf9 cell extracts and then affinity purified by binding to and eluting from purified LEF-11 protein (Monsma & Wolfner, 1988). At 18 h p.i., LEF-11 was detected within large and dense nuclear regions, commonly observed in AcMNPV-infected Sf9 cells. A low intensity fluorescence signal in infected cells appeared to localize to the expanded nuclei of infected cells (Fig. 3B). LEF-11 does not appear to be expressed at high levels in infected cells, as indicated by comparing lef-11 mRNAs to those of overlapping transcripts from the pp31 gene (Fig. 1D). Because of its reduced abundance, detection of LEF-11 in infected cells was difficult and required the use of affinity purified antibodies. LEF-11 appeared to be generally localized to dense regions of the nucleus. Based on transient assays for late transcription (Lu & Miller, 1995), potential roles for LEF-11 might include interactions with regulatory DNA sequences or the core late RNA polymerase, or both. Discrete sites of Bombyx mori nucleopolyhedrovirus (BmNPV) DNA replication within the nucleus were characterized recently and several BmNPV proteins associated with DNA replication (IE1, DBP and LEF-3) were found to localize to sites referred to as ‘replication factories’ (Okano et al., 1999). It is likely that late transcription occurs on newly replicated viral DNA within or
AcMNPV LEF-11 expression

Fig. 3. (A) Western blot detection of LEF-11 in infected cell extracts at various times p.i. Numbers above the lanes represent the time (h) p.i. when cell lysates were harvested. (Sf9, mock-infected cells; LEF-11, purified LEF-11 expressed from E. coli; Xf, Sf9 cells transfected with a plasmid expressing LEF-11; M, protein size standard markers). Infected cell lysates were prepared from Sf9 cells infected with AcMNPV (m.o.i. = 10) and LEF-11 proteins were detected using affinity purified anti-LEF-11 antibodies (1:200 dilution) as a primary antibody and a goat anti-rabbit IgG–alkaline phosphatase conjugate as a secondary antibody (1:10000 dilution). The anti-LEF-11 antiserum was generated against His-tagged LEF-11 expressed in E. coli and purified by metal-affinity chromatography. Anti-LEF-11 antibodies were affinity purified by binding and eluting from NitroPure membranes containing the LEF-11 fusion protein, as described earlier (Monsma & Wolfner, 1988). (B) Immunofluorescent detection of LEF-11 in AcMNPV-infected Sf9 cells. Sf9 cells were infected with AcMNPV (m.o.i. = 100) and fixed in methanol at 18 h p.i. Cells were then examined for LEF-11 localization using affinity purified anti-LEF-11 polyclonal antibodies (1:200 dilution) and an Alexa Fluor 594-conjugated secondary antibody. Panels (i) and (iii) show light micrographs and panels (ii) and (iv) show epifluorescence images of immunostained cells. LEF-11-specific staining (arrowheads) within dense nuclear structures in infected cells is shown. Nuclei were identified by phase contrast microscopy (i, iii). Sf9, mock-infected cells; AcMNPV 18 h p.i., infected cells at 18 h p.i.

near such sites and late transcription factors might be expected to localize to the same or nearby sites within the infected cell nucleus. Additional studies will be necessary to determine whether LEF-11 and other proteins that may be associated with late transcription are localizing to the same nuclear regions identified as replication factories.

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