Identification and Transcriptional Regulation of the Baculovirus *lef-6* Gene

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We identified an *Autographa californica* nuclear polyhedrosis virus (AcMNPV) gene, the late expression factor 6 gene (*lef-6*), which is involved in expression from late and very late AcMNPV gene promoters but not from an early AcMNPV gene promoter in transient expression assays. This gene was located within the *Pst*I I fragment of the AcMNPV genome (14.7 to 17.9 map units), immediately downstream of *Ac-iap*, the AcMNPV homolog of a baculovirus gene family involved in blocking apoptotic programmed cell death. The nature and temporal regulation of both *Ac-iap* and *lef-6* transcripts was examined. *Ac-iap* and *lef-6* were cotranscribed as bicistronic messages at both early and late times postinfection. In addition, *lef-6* was transcribed as a monocistronic mRNA by initiation from an early promoter within *Ac-iap*.

Upon infection of insect cells with *Autographa californica* nuclear polyhedrosis virus (AcMNPV), viral genes are expressed in a coordinated temporal fashion. Early genes are thought to be transcribed by the host RNA polymerase II (16, 19, 21) in the absence of newly synthesized protein synthesis or viral DNA replication (9, 11, 20, 23, 30, 36, 40, 42). In contrast, late and very late genes appear to be transcribed by an α -amanitin-resistant virus-induced RNA polymerase activity (12, 13, 16, 21). Transcription of late and very late genes is contingent on viral DNA replication and newly synthesized proteins (11, 20, 36, 39, 45).

To direct a late temporal pattern of expression during infection, late promoters require only the sequence TAAG and possibly a few flanking nucleotides (26, 27, 34), but activation of late promoters requires the expression of a number of viral genes. Several of the AcMNPV genes involved in late and very late gene expression have been identified by characterizing temperature-sensitive mutants defective in early or late gene expression and/or DNA replication (i.e., *p143*, *p47*, and *ie-1*) (14, 25, 29, 35). Five more genes have been identified recently by using a transient expression assay (23, 30–32) in which all *trans*-acting factors required for late or very late promoter-directed reporter gene expression are provided by an overlapping library of AcMNPV genomic clones. Specific genes required for reporter gene expression are identified by replacing the large AcMNPV library clones with subclones containing single *trans*-activating genes.

In this study, we have used this assay to identify another AcMNPV gene involved in expression from the late major capsid protein (*vp39*) and very late polyhedrin (*polh*) gene promoters but not from the early *etl* promoter. This gene, designated the late expression factor 6 gene (*lef-6*), was previously sequenced (open reading frame [ORF] 14 in reference 5). The translational initiation codon for this ORF is located only 4 nucleotides (nt) downstream of the termination codon of *Ac-iap*, a homolog of two other baculovirus genes

that block apoptosis (3, 8, 10). We also report the transcriptional mapping of *lef-6* and *Ac-iap*.

MATERIALS AND METHODS

Cells and virus. AcMNPV L-1 (22) was propagated in the IPLB-SF-21 (SF-21) cell line derived from *Spodoptera frugiperda* (43) and grown at 27°C in TC-100 medium (GIBCO BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum and 0.26% tryptose broth (28).

DNA cotransfections and CAT assays. DNA was introduced into SF-21 cells by calcium phosphate coprecipitation (28, 32), and cell lysates assayed for chloramphenicol acetyltransferase (CAT) activity (15).

Reporter plasmids pETCAT^{thr5}, pCAPCAT, and phcwt contain the CAT gene under early *etl*, late *vp39*, or very late *polh* gene promoter control, respectively (27, 32, 41). Plasmid pBCNE contains *lef-1* (30). Plasmid pPstI-I contains the AcMNPV *Pst*I I (14.7 to 17.9 map units [m.u.]) fragment cloned into the *Pst*I site of pBluescript. A subclone of pPstI-I, pPstIRV, lacks a 2.1-kbp fragment from the *Pst*I site at 14.7 m.u. to a unique *EcoRV* site (16.3 m.u.) within *Pst*I-I. pAcIAP-Ps was constructed by deleting a 1.2-kbp fragment from pPstIRV between the *EcoRV* site (16.3 m.u.) to a unique *Mlu*I site in *Pst*I-I at 17.3 m.u. Plasmid pAcIAP-Ps/*Nsi*I was constructed by digesting pAcIAP-Ps with *Nsi*I, a unique site within *Pst*I-I, blunt ending with T4 DNA polymerase, and religating. This created a frameshift mutation in which 4 nt, TGCA (nt 11287 to 11290 in reference 5), were deleted from *Ac-iap*, but the *lef-6* ORF downstream of *Ac-iap* remained intact. pAcIAP contains *Ac-iap*, 351 nt upstream of the first methionine of *Ac-IAP*, and an incomplete *lef-6* ORF. pAcIAP was constructed by digesting pPstIRV with *Mlu*I, blunt ending with the Klenow fragment of *Escherichia coli* DNA polymerase I (GIBCO BRL), then digesting with *Sal*I (a site within *lef-6*), and cloning a 1.3-kbp *Mlu*I-to-*Sal*I fragment into the *EcoRV* and *Sal*I sites of pBluescript. Plasmid pAcIAP-Ps Δ el, containing a 329-bp deletion within *Ac-iap*, was constructed by digesting pAcIAP-Ps with *Nsi*I, repairing the ends with T4 DNA polymerase, cutting with *Nru*I, and religating. pAcIAP- Δ elHX Δ elSal, containing a 198-bp deletion within *lef-6*, was made by first deleting a *Sal*I site in the polylinker region of pAcIAP-Ps by removing a *Hind*III-to-*Xho*I fragment, blunt ending the ends with the Klenow fragment, and religating to

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construct pAcIAP-PsdelHX. Then, a *SalI* fragment within *lef-6* in pAcIAP-PsdelHX was deleted. A plasmid containing only the *lef-6* ORF and the C terminus of *Ac-iap* to include a promoter region for *lef-6* was constructed. This plasmid, pPstIRV/BglI-Ps, was created by cloning a 757-bp fragment of pPstIRV, from a unique *BglI* site, which was blunt ended, to the *PstI* site at 17.9 m.u., into pBluescript at the *EcoRV* and *PstI* sites, respectively.

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated (6) from SF-21 monolayers either mock or AcMNPV infected at a multiplicity of infection of 20 PFU per cell. Cycloheximide (100 µg/ml) or aphidicolin (5 µg/ml) was added to specific samples as described previously (36) except that cycloheximide pretreatment was for 30 min.

RNAs complementary to the *lef-6* or the *Ac-iap* message were prepared by in vitro transcription, using T7 RNA polymerase (Stratagene, La Jolla, Calif.) in the presence of [³²P]UTP, and used as probes for Northern blot analyses as described previously (2). A *lef-6*-specific RNA probe was prepared by using p*lef-6*/Sal, which contained a 179-bp *SalI* fragment derived from the *lef-6* ORF. To detect *Ac-iap* transcripts, a 329-base RNA probe was prepared from pAc-IAP-PsdelNsi-Ps which contained a 737-bp insert from a unique *MluI* site at approximately 16.4 m.u. to a unique *NsiI* site within the AcMNPV *PstI* I fragment in pBluescript KS+ (Stratagene). The 329-base probe was synthesized from the *NsiI* site to a single *NruI* site within the 737-bp insert.

S1 nuclease protection analyses. A 5'-end probe was prepared from pAcIAP-PsdelHX containing an AcMNPV fragment extending from a *MluI* site at approximately 16.4 m.u. to a *PstI* site at 17.9 m.u. The plasmid was digested with *XbaI* and labeled with T4 polynucleotide kinase (GIBCO BRL) and [^γ-³²P]ATP. A 4.4-kbp fragment was obtained by digesting at an *SstI* site located within vector sequences and gel isolating this fragment. For S1 reactions (44), samples of total RNA (30 µg) and labeled fragment were dried, resuspended in 30 µl of formamide-piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer (80% formamide, 40 mM PIPES [pH 6.4], 0.4 M NaCl, 1 mM EDTA), denatured for 10 min at 100°C, and hybridized overnight at 37°C. After hybridization, S1 nuclease (100 U per reaction) in S1 buffer (5 mM sodium acetate, 4.5 mM ZnSO₄, 280 mM NaCl, 5% glycerol, 20 µg of denatured salmon sperm DNA per ml) was added to each reaction mixture, and the mixtures were incubated for 30 min at room temperature. The S1 nuclease-resistant fragments were chilled on ice 5 min, proteins were phenol-chloroform extracted, and DNA was precipitated before denaturation and separation in polyacrylamide-urea gels.

The same plasmid, pAcIAP-PsdelHX, was also used to map the 3' ends of *lef-6* transcripts. Plasmid DNA was cut with *SalI* and 3' end labeled with T4 DNA polymerase in the presence of [^α-³²P]dCTP as described previously (37). *SalI*-digested DNA was then cut within the multicloning site with *KpnI*, and a 3.3-kbp fragment was gel isolated. Hybridization and S1 nuclease digestions were carried out as described above except that hybridization was performed at 54°C and S1 nuclease digestion was carried out at 37°C.

Primer extension analyses. A 15-nt oligomer (5'-TCGA CGCTGTTTTTC-3') complementary to the *lef-6* ORF and starting at the 5'-proximal *SalI* site within *lef-6* was used to map the 5' ends of *lef-6* transcripts by primer extension analysis. To map the 5' ends of *Ac-iap* RNAs, the primer IAP2 (5'-GTCAGACATTTTAGCTCGTTAAAGTTAC-3'), complementary to nt -1 to -19 with respect to the ATG of *Ac-IAP* (in boldface), was used in primer extension reactions. Primers were radiolabeled at the 5' end by using T4 polynucleotide

kinase. Primers (10 ng per reaction) and total RNA (15 µg per reaction) were hybridized overnight in 0.2 M NaCl and 5 mM PIPES (pH 6.4) at 51 or 43°C to map the *lef-6* and *Ac-iap* 5' ends, respectively. Annealed primers were extended with Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). Extension products were denatured, separated in polyacrylamide-urea gels, and visualized by autoradiography.

RESULTS

Identification of *lef-6*. Using the transient expression assay designed to identify genes specific for expression from a major late AcMNPV promoter, *vp39* (32), we have searched for genes necessary for late gene expression in a region of AcMNPV which contained three overlapping clones, BC5, HL8, and HL5, from the overlapping set of AcMNPV clones which support late gene expression (30). Although the ends of these three clones are not known precisely, they overlap in the region between 14.2 and 18.4 m.u. (Fig. 1A).

The region of the genome contained in the BC5 clone (starting between 4.8 and 4.9 m.u. and ending between 14.7 and 18.4 m.u.) is known to contain a gene, *lef-1* (7.5 to 8.65 m.u.), required for significant expression from the *vp39* promoter (30). As shown in Fig. 1B (compare lane 1 or 3 with lane 2), omission of BC5 from the clone library eliminates expression from the pCAPCAT reporter plasmid, but replacement of BC5 with a clone containing only *lef-1*, pBCNE, restores expression from the *vp39* promoter in this reporter plasmid.

As previously shown (30), expression from the *vp39* promoter is not drastically affected if HL8 (endpoints between 7.35 to 13.1 and 22.0 to 23.4 m.u.) or HL5 (endpoints between 14.2 to 14.7 and 23.4 to 29.0 m.u.) are omitted from the complementing set of clones (Fig. 1B; compare lane 1 with lanes 4 and 5). When the two neighboring clones BC5 and HL8 or HL8 and HL5 were simultaneously omitted from the late gene expression-supporting library of clones, expression from the late promoter again was not drastically affected (Fig. 1B; compare lane 1 with lanes 6 and 7). Also, omission of BC5 and HL5, the clones extending toward the left and right, respectively, of this region encompassed by the three library clones, did not dramatically affect expression (Fig. 1B, lane 8). However, simultaneous omission of the three library clones, BC5, HL8, and HL5, reduced expression substantially (Fig. 1B, lane 9). Thus, the gene(s) necessary for expression from the *vp39* promoter seemed to be contained within all three library clones, although there was the possibility that there were at least two functionally redundant genes in this region. The slight decrease (two- to fivefold) in late gene expression which is sometimes observed upon omission of one or two of these clones and in the presence of *lef-1* (pBCNE) may be due to a gene copy number effect (i.e., additional copies of the gene in question were supplied by BC5, HL8, or HL5).

To identify the specific gene(s) necessary for expression from the *vp39* promoter in this region, we replaced BC5, HL8, and HL5 with pBCNE, to supply *lef-1*, and pPstI-I (Fig. 1A), a clone containing the *PstI* I fragment which spans most of the overlap among the three clones. This clone was able to reconstitute CAT activity in the presence of the remaining set of AcMNPV clones and pBCNE (Fig. 1B; compare lanes 1 and 10). Subclones of pPstI-I, pPstIRV and pAcIAP-Ps, were also able to substitute for the lack of BC5, HL8, and HL5 (Fig. 1, lanes 11 and 12). Plasmid pAcIAP, which contained the *Ac-iap* (10) and other smaller overlapping ORFs, was not able to supply the gene(s) necessary for significant expression from the *vp39* promoter (Fig. 1B, lane 13). This result was confirmed by testing pAcIAP-Psdel, which contained a deletion between the

*Nru*I and *Nsi*I sites of *Ac-iap* disrupting ORFs 6-3 and 4-3 as well as conserved repeat BIR-1 in *Ac-iap* (3) but contained the region upstream of and the region downstream of *Ac-iap* including *lef-6*. This plasmid was able to support full expression (Fig. 1B, lane 14). Similarly, a plasmid with an out-of-frame mutation within *Ac-iap*, pAcIAP-Ps/*Nsi*I, supported expression in the assay (Fig. 1B, lane 15). Plasmid pAcIAPdelHXdelSal, similar to pAcIAP-Ps but containing a 179-bp deletion which disrupted *lef-6* and two smaller overlapping ORFs, ORFs 4-2 and 6-1, was not able to restore *trans*-activating function in this assay (Fig. 1B, 16). Thus, one of these three ORFs was necessary for late gene expression. The plasmid containing the smallest insert which was able to restore expression in the absence of BC5, HL8, and HL5 was pPstIRV/*Bgl*I-Ps (Fig. 1B, lane 17).

We also tested a plasmid, pIAPZ, similar to pPstIRV in which the region between the *Nru*I and *Aat*II sites within *Ac-iap* was replaced with *E. coli lacZ* cassette in which *lacZ* is under *Drosophila melanogaster hsp70* promoter control and terminated by a simian virus 40 polyadenylation signal (Fig. 1A). This plasmid was not able to restore expression (data not shown). We can account for the inactivity of this plasmid on the basis of our further characterization of transcription in this region; the region of *Ac-iap* between the *Bgl*I and *Aat*II sites contains the start site for early transcription of *lef-6* (Fig. 3D). Thus, the deletion in pIAPZ removed the early *lef-6* promoter and start sites, disrupting transcription of *lef-6*. Furthermore, the same *lacZ* cassette, when introduced in either orientation further upstream between the *Nru*I and *Nsi*I sites within *Ac-iap*, did not disrupt *lef-6* activity (data not shown). The effect of these *lacZ* insertions indicated that *lef-6*, rather than the antisense ORFs 6-1 and 4-2, was responsible for the activity in this region. We have designated this *trans*-activating gene *lef-6* (Fig. 1A).

Role of *lef-6* in expression from the early *etl* and very late *polh* gene promoters. To determine if *lef-6* was affecting early or very late gene expression, we tested the effects of the presence or absence of *lef-6* on expression from the early *etl* or very late *polh* promoter, using pETCATHr5 or phcwt, respectively, as the reporter plasmid. The removal of BC5, HL8, and HL5 from the set of AcMNPV clones in the presence or absence of *lef-1* (pBCNE) and *lef-6* (pPstIRV/*Bgl*I-Ps) did not affect expression from this early promoter (Fig. 2A), confirming previous results that *ie-1* (94.7 to 96.9 m.u.) is sufficient for substantial expression from the *etl* promoter (32). In contrast, expression from the very late *polh* promoter required *lef-6* for efficient very late gene expression in this assay (Fig. 2B).

Transcriptional mapping of the 5' ends of the *lef-6* RNAs. We were interested in investigating the transcriptional pattern and regulation of *lef-6*. The translational start codon (ATG) of LEF-6 and the translational stop codon (TAA) of Ac-IAP are separated by only 4 bp, TAATAAAATG, overlapping a potential polyadenylation signal (in boldface).

The 5' ends of *lef-6* were mapped by both primer extension analysis and S1 nuclease protection assays. An oligonucleotide complementary to 15 nt spanning the *Sal*I site positioned 94 bp downstream of the first and only methionine in LEF-6 was used to reverse transcribe RNAs extracted from mock- or AcMNPV-infected SF-21 cells harvested at specific times postinfection (p.i.). Two main polymerase extension products of 178 and 165 nt, mapping to -85 and -71 nt with respect to the methionine, were clearly seen (Fig. 3B). The two extension products had similar temporal kinetics of expression. The larger extension product mapped to a CAGT motif (Fig. 3B and D), a common start site in some but not all early baculovirus transcriptional start sites (4, 7, 17, 18, 38), although a consensus TATA box is not present 30 nt upstream. Both extension products present at 3, 6, 9, and 12 h p.i. but absent at 24 and 48 h p.i. were virus specific. These products were also observed with RNAs prepared from cells treated with either the protein synthesis inhibitor cycloheximide or the DNA synthesis inhibitor aphidicolin at 9 and 12 h p.i. (Fig. 3B). Transcription was slightly enhanced in the presence of cycloheximide and slightly reduced in the presence of aphidicolin at 9 and 12 h p.i.

To map the 5' termini of *lef-6* RNAs by S1 nuclease protection analysis, we used a 4.4-kbp DNA fragment which was 5' end labeled at an *Xba*I site (Fig. 3A) and extended through the *Mlu*I site upstream of *Ac-iap* into vector sequences. Two fragments of approximately 225 and 210 nt (Fig. 3C, thin arrows), mapping just downstream of the *Bgl*I site (Fig. 3C, lane P/*Bgl*I) were observed throughout the time course except at 24 and 48 h p.i. (Fig. 3C). These protected fragments were also observed at 9 and 12 h p.i. in the presence of inhibitors. These results are consistent with those obtained in primer extension experiments; the distances between the 5'-end-labeled *Xba*I site and the start sites mapped by primer extensions are 219 and 206 nt.

The S1 nuclease protection assays also showed a number of large unresolved protected fragments (Fig. 3C, thick arrow) present from 6 h p.i. and in the presence of cycloheximide but not in the presence of aphidicolin. Since these transcripts mapped upstream of the *Nsi*I site (Fig. 3C, lane P/*Nsi*I), they probably originated from the *Ac-iap* promoter.

Mapping the 5' termini of *Ac-iap* transcripts. The 5' ends of *Ac-iap* RNAs were mapped by primer extension analysis using an oligomer complementary to 19 nt immediately upstream of the first methionine of Ac-IAP. Two extension products, spaced 1 nt apart (Fig. 4A, arrows e), were apparent for RNAs isolated at 9 and 12 h p.i. in the presence or absence of cycloheximide but were not observed in RNA isolated from aphidicolin-treated cells. These products were still apparent at 24 and 48 h p.i., albeit at lower levels. At least two major (Fig. 4A, arrows a and d) and two minor products (Fig. 4A, arrows b and c) accumulated at 9 h p.i., became more abundant at 12 and 24 h p.i., and decreased slightly by 48 h p.i. These products

FIG. 1. Distribution of ORFs in the AcMNPV *Pst*I-I (14.7 to 17.9 m.u.) genomic region and the identification of *lef-6*. (A) The AcMNPV library clones BC5, HL8, and HL5 which overlap the *Pst*I-I region are shown below an *Eco*RI restriction map of a portion of the AcMNPV genome. The dashed line box contains an enlargement of the *Pst*I-I restriction map and illustrates organization of ORFs larger or equal to 50 amino acids. The open arrows show the direction and size of each ORF obtained from the sequence reported by Braunagel et al. (5). Plasmids or AcMNPV library clones tested in transient expression assays are indicated below the restriction maps, and a + or - sign at the right indicates whether they restored CAT activity. (B) CAT activity from the reporter plasmid pCAPCAT in the presence of the entire AcMNPV clone library (lane 1) or in the absence of specific library clones indicated below each lane (lanes 2 to 17). The presence (+) or absence (-) of *lef-1* supplied by plasmid pBCNE is indicated above each lane. Additional plasmids added in cotransfections with the reporter plasmid are indicated above each lane. The relative CAT activity compared with that in lane 1 is shown below each reaction. To quantitate CAT activity, the protein lysates were diluted so that the reactions were within the linear range. The results are representative of several independent experiments. The positions of migration of the unacetylated substrate (Cm) and acetylated products (Ac Cm) of the enzymatic reactions are indicated at the right.

were not observed in the presence of cycloheximide or aphidicolin, indicating they were late transcripts. The two major late extension products (Fig. 4A, arrows a and d) map to two TAAGs present at -188 and -408 with respect to the methionine of Ac-IAP. Extension product d maps to the first A in the TAAG sequence motif (Fig. 4A and B), but because of the large size of product a, it is difficult to ascertain the exact nucleotide where this extension product terminates. The ATGs for ORFs 1-3 and 2-8 lie between the late start sites and the ATG of Ac-*iap* (Fig. 1A). Thus, it is possible that these late transcripts are polycistronic. Figure 4B summarizes the approximate positions where all of the observed extension products terminated.

Transcriptional mapping of the 3' ends of *lef-6* RNAs. The 3' termini of *lef-6* RNAs were mapped by using a 3.3-kbp fragment 3' end labeled at the second *SalI* site within *lef-6* and extending past the *PstI* site at 17.9 m.u. into vector sequences (Fig. 5A). At least two bands, approximately 240 and 255 nt in length, were observed in the autoradiogram (Fig. 5B, thick arrows). These protected fragments were observed throughout the time course of infection and in the presence of protein and DNA synthesis inhibitors. Two potential polyadenylation signals (AATAAA) located 224 and 251 nt downstream of the 3'-end-labeled *SalI* site and 7 and 34 nt downstream of the LEF-6 termination codon may account for the presence of these protected fragments. Upon longer exposures of the autoradiogram, a 359-nt band was observed at 24 and 48 h p.i. (Fig. 4B, thin arrow). This 359-nt protected fragment seen at late times comigrates with the *SalI*-labeled S1 nuclease probe digested at the AcMNPV and vector sequence junction (i.e., *PstI*), suggesting that some late transcripts traversing this region may not be polyadenylated at either AATAAA site. Since only early transcripts which did not persist at late times initiated from the *lef-6* promoter within the Ac-*iap* ORF (Fig. 3B), late transcripts terminating at these two AATAAA sites are likely those late RNAs observed by 5' S1 nuclease analysis (Fig. 3C) to initiate further upstream, probably from the Ac-*iap* promoter (Fig. 4). Furthermore, there are no TAAGs present on the DNA strand containing these ORFs between TAAGs upstream Ac-*iap* and the polyadenylation signals downstream of *lef-6*.

Northern blot analysis of *lef-6* transcripts. We determined the temporal regulation and overall size of *lef-6* RNAs by Northern blot analysis. Three RNAs of approximately 1.8, 1.6, and 0.95 kb were observed when the blot was hybridized with a *lef-6*-specific RNA probe (Fig. 6A and B). The 0.95-kb RNA was observed at 3 h p.i., was maximal at 9 h p.i., declined slightly at 12 h p.i., and was not observed at later times (Fig. 6B, arrow c). The 0.95-kb RNA was the only one of the three transcripts observed in the presence of both cycloheximide and aphidicolin, indicating it is an early transcript which persists in the presence of other early gene products. The pattern of expression of this RNA was consistent with the pattern of expression observed by primer extension and 5' S1 analyses for *lef-6* RNAs initiating from within the Ac-*iap* ORF (Fig. 3B and C, thin arrows). The predicted size of transcripts initiating at the CAGT upstream of *lef-6* and terminating at the polyadenylation site just downstream of the *lef-6* termination codon (Fig. 6A) is 650 bases, in agreement with the determined size of a 0.95-kb polyadenylated RNA.

The 1.6-kb transcript (Fig. 6B, arrow b) was observed at early times and in the presence of cycloheximide but was not apparent in the presence of aphidicolin. This pattern of expression was similar to that of the early transcripts detected in primer extension analysis of RNAs initiating upstream of Ac-*iap* (Fig. 4A, arrows e). The distance between this early

FIG. 2. Effect of *lef-6* on expression from reporter plasmids containing either the early *etl* (A) or very late *polh* (B) promoter. The complete AcMNPV clone library was cotransfected with the reporter plasmid in lanes 1, and the AcMNPV library lacking clones BC5, HL8, and HL5 was cotransfected in lanes 2 to 5. Additional plasmids are indicated above each lane. The positions of migration of acetylated (Ac Cm) and unacetylated (Cm) chloramphenicol are indicated at the right.

start site and the polyadenylation site downstream of *lef-6* is 1.52 kb, consistent with the estimated size of this 1.6-kb RNA. We also mapped a late transcript initiating at a TAAG site 48 nt upstream of the early start site by primer extension analysis (Fig. 4); these late RNAs may comigrate with the early 1.6-kb RNA in Northern blots, thus explaining the increased levels of expression seen in the 1.6-kb RNA at 24 h p.i. in Northern blot analysis (Fig. 6B, arrow b).

The RNA of approximately 1.8 kb was apparent only at late

FIG. 3. Mapping the 5' termini of *lef-6* transcripts. (A) The labeled fragment used to map the 5' ends of *lef-6* transcripts by S1 nuclease protection analyses is indicated above the *lef-6* and *Ac-iap* ORFs, represented as arrows above a restriction map of this region. The asterisk shows the position (*Xba*I site) at which the fragment was 5' end labeled. The lines below the ORFs indicate the sizes and locations of the protected fragments observed. The broken line indicates the ambiguity in the size of one protected fragment. The number in parentheses indicates the map units at the *Pst*I site. (B) Primer extension reactions using total RNA extracted from mock-infected (mi) or AcMNPV-infected (lanes 3 to 48) SF-21 cells at the times in hours shown above each lane. Cells were treated with cycloheximide in lanes 9C and 12C and with aphidicolin in lanes 9A and 12A, and were harvested at 9 or 12 h p.i. An oligonucleotide complementary to sequences starting at the first *Sal*I site within *lef-6* was used as a primer. Lanes G, A, T, and C are dideoxyribonucleotide sequencing reactions using the same primer and a plasmid containing this region. Numbers at the right indicate the sizes of labeled fragments used as size markers (see panel C). (C) S1 nuclease protection analysis of *lef-6* transcripts. In lanes P/BglI, P/NsiI, and P/KpnI, the 4.4-kbp labeled fragment labeled at the *Xba*I site (see panel A) was digested with BglI, NsiI, and KpnI, respectively, to provide size markers. Lane P contains the untreated labeled probe. Other lane designations are the same as for panel B. The arrows at the right point to specific protected fragments discussed in the text, and the numbers at the left indicate the migration of radiolabeled markers in nucleotides. (D) The nucleotide sequence from -35 to -113 with respect to the A in the ATG of LEF-6 is shown. Key restriction sites (in boldface) are indicated at the ends above the nucleotide sequence. The asterisks show the positions of transcriptional start sites determined by primer extension analysis. The early baculovirus transcriptional start site, CAGT, is underlined.

FIG. 4. Primer extension analysis of *Ac-iap*-specific transcripts. (A) An oligomer complementary to the first 19 nt upstream of the *Ac-IAP* translational start site was used as a primer for reverse transcription of RNA from mock- or *AcMNPV*-infected SF-21 cells. Lane designations are the same as described in the legend for Fig. 3B. The letters and arrows at the left indicate the positions of primer extension products described in the text; the positions of radiolabeled markers are indicated at the right in nucleotides. (B) The nucleotide sequence (5) from -108 to -421 of *Ac-iap* with respect to the first ATG shows the nucleotide positions (asterisks) to which the 5' ends of the late and early transcripts d and e, respectively, map. The approximate positions of extension products of late transcripts (a to c in panel A) are indicated by + signs. The TAAG or CAGT sequence motifs are in boldface or underlined, respectively. The *MluI* site is in boldface and labeled.

times p.i. (e.g., 12 and 24 h p.i.), and its steady-state levels decreased by 48 h p.i. The temporal regulation and size of this late 1.8-kb RNA correspond to the largest late extension products from RNAs initiating upstream of *Ac-iap* (Fig. 4A, arrows a, b, and c).

Northern blot analysis of *Ac-iap* transcripts. A strand-specific RNA probe extending from the *NsiI* to the *NruI* sites within *Ac-iap* hybridized two RNAs on Northern blots which correspond in approximate size and temporal regulation to the 1.6- and 1.8-kb RNAs observed with the *lef-6*-specific probe. The smaller of the two RNAs was observed by 3 h p.i. and was

FIG. 5. Mapping the 3' termini of *lef-6* transcripts. (A) The 3.3-kbp labeled fragment used to map the 3' ends of *lef-6* RNAs is shown at the top, with an asterisk denoting the position of the radiolabeled 3' end (*SalI* site). The *Ac-iap* and *lef-6* ORFs are indicated by open arrows. The sizes and locations of protected fragments are shown below the *lef-6* ORF, above a restriction map of the region. (B) An S1 nuclease protection assay showing the protected products (arrows). Lane designations are the same as for Fig. 3B. Lane P is the untreated probe, and in lane P/*PstI*, the probe was digested at the junction of vector and *AcMNPV* sequences with *PstI*. Markers are indicated at the left in nucleotides.

sensitive to aphidicolin but not cycloheximide, similar to the 1.6-kb RNA in Northern blot hybridized to a *lef-6*-specific probe (Fig. 6B, arrow b) and in primer extension analyses using an *Ac-iap*-specific primer (Fig. 4A, arrows e). Again, the increase in steady-state levels of this RNA at 24 h p.i. may correspond to a comigrating late transcript of approximately the same size (see above). The larger of the two RNAs was a late RNA present at 12, 24, and 48 h p.i. and sensitive to both aphidicolin and cycloheximide. This RNA was similar in temporal expression and size to the 1.8-kb RNA observed with the *lef-6*-specific probe and probably corresponds to the distal

FIG. 6. Northern blot analyses of *lef-6* and *Ac-iap* transcripts. (A) Summary of transcriptional mapping analysis of *lef-6* and *Ac-iap* and the locations of probes used for hybridization in Northern blot analyses. The directions and sizes of transcripts are shown by arrows above the ORFs (open arrows). The L or E at the beginning of each transcript indicates whether it is present at early (E) or late (L) times p.i. The broken arrow at the end of the late transcripts indicates inefficient termination of late transcripts at the end of the *lef-6* ORF. The locations and directions of strand-specific RNA probes used in Northern analysis of *lef-6* (arrow A) and *Ac-iap* (arrow B) are indicated below the ORFs and above a restriction map of this region. The number in parentheses refers to the map units of the *PstI* site. (B) Northern blot hybridized to the *lef-6* probe (arrow A of panel A). (C) Northern blot analysis using an *Ac-iap*-specific probe (arrow B in panel A). Letters and arrows at the right illustrate the positions of different transcripts. Lane designations are as described in the legend to Fig. 3B. Numbers at the left are molecular size markers in kilobase pairs.

TAAG-initiated transcript originating upstream of *Ac-iap* and terminating downstream of *lef-6*.

DISCUSSION

The gene functionally identified in this study, *lef-6*, is the ninth gene found to be involved in expression from the *vp39* and *polh* promoters (23, 30–32). These genes may function either as *trans*-acting transcriptional or translational activators or as primary or accessory replication factors, since late gene expression requires DNA replication. The *lef-6* sequence predicts a product of 19,969 Da with no significant overall homology to other sequences in the data bases. There is some sequence similarity of LEF-6 to the C-terminal end of a 19-kDa DNA-dependent RNA polymerase subunit of vaccinia virus (1) over a stretch of 42 amino acids, but this similarity is very limited and may be coincidental. The *lef-6* ORF contains a possible nuclear targeting sequence, KRPRR, from residues 100 to 104.

Transcriptional analyses of *lef-6* revealed an early 0.95-kb monocistronic RNA initiating within *Ac-iap* and both early and late 1.6-kb bicistronic RNAs originating from promoters upstream of *Ac-iap* and overlapping the early *lef-6* monocistronic RNA entirely. A late 1.8-kb RNA which may be polycistronic, specifying ORFs 1–3 and 2–8 (Fig. 1A) as well as *Ac-iap* and *lef-6*, also appears to overlap entirely with the 0.95-kb *lef-6*-specific RNA. Although we did not perform 3' S1 analysis using a probe labeled within *Ac-iap* and terminating past the *Ac-iap* ORF, we saw no evidence of *Ac-iap*-specific transcripts terminating at the polyadenylation site overlapping the *Ac-iap* termination codon by Northern blot analysis. The expected size of these *Ac-iap*-specific transcripts would be between 1.0 and 1.3 kb, depending on which of the start sites upstream of *Ac-iap* was used.

The pattern of *lef-6* transcription is reminiscent of but not identical to *cg30* transcription (40). Early *cg30* monocistronic RNAs originate from a promoter within its upstream ORF, *vp39*. At late times, *cg30* is transcribed from *vp39* promoters as part of a bicistronic RNA. Early *vp39*-*cg30* bicistronic RNAs, however, were not observed. Although *lef-6* and *Ac-iap* are cotranscribed, it is not known whether *lef-6* is translated from the bicistronic RNAs; *cg30* does not appear to be translated from the late *vp39*-*cg30* bicistronic RNAs (33).

The early *lef-6* monocistronic and *Ac-iap*-*lef-6* bicistronic messages were differentially regulated. The monocistronic mRNA was most abundant at 9 h p.i., was stimulated by cycloheximide treatment, and persisted in the absence of DNA replication. The bicistronic early mRNA was more abundant about 12 h p.i. and was transcribed in the presence of cycloheximide but was not stimulated by this treatment. These *Ac-iap*-*lef-6* RNAs either are not transcribed in the presence of aphidicolin or are rapidly degraded by early gene products present in aphidicolin-treated but not cycloheximide-treated cells. Differences in sensitivity to cycloheximide or aphidicolin are not novel to this region of the genome. A similar pattern of expression was also observed for some transcripts of the DNA polymerase gene (42) and for *lef-1* (30). Whether this pattern of expression is associated with a novel mechanism of regulation is not known.

Omission of the three library clones BC5, HL8, and HL5 eliminates all of the sequences within *PstI*-G (7.3 to 13.4 m.u.) from the clone library as well as other sequences. Another laboratory reported that *ie-1* and two ORFs within *PstI*-G were sufficient to *trans* activate expression from another promoter (18a). Although this promoter was described as a late promoter, the properties of the promoter have not been described

in detail and the expression observed may not be due to late TAAG-initiated promoter activity. Also relevant is the fact that one of the two *Pst*I-G ORFs reported to be required for expression was deleted from the genome without obvious effect on general viral gene expression or viral infectivity in cell culture and in vivo (28a). Although we have not excluded the possibility that another gene with a function redundant to that of *lef-6* is also present in this region, expression from the late *vp39* and very late *polh* promoters can be fully supported by deleting BC5, HL8, and HL5 from the library and supplying subclones containing *lef-1* and *lef-6*.

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REFERENCES

- Ahn, B.-Y., J. Rosel, N. B. Cole, and B. Moss. 1992. Identification and expression of *rpo19*, a vaccinia virus gene encoding a 19-kilodalton DNA-dependent RNA polymerase subunit. *J. Virol.* **66**:971-982.
- Berger, S. L., and A. R. Kimmel (ed.). 1987. Guide to molecular cloning techniques. Methods in Enzymology. Academic Press, Inc., New York.
- Birnbaum, M. J., R. J. Clem, and L. K. Miller. 1994. An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a peptide with Cys/His sequence motifs. *J. Virol.* **68**:2521-2528.
- Blissard, G. W., and G. F. Rohrmann. 1989. Location, sequence, transcriptional mapping, and temporal expression of the gp64 envelope glycoprotein gene of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. *Virology* **170**:537-555.
- Braunagel, S. C., K. D. Daniel, L. M. Reilly, L. A. Guarino, T. Hong, and M. D. Summers. 1992. Sequence, genomic organization of the *Eco*RI-A fragment of *Autographa californica* nuclear polyhedrosis virus, and identification of a viral-encoded protein resembling the outer capsid protein VP8 of rotavirus. *Virology* **191**:1003-1008.
- Chirgwin, J. M., A. E. Przybyla, R. J. McDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299.
- Chisholm, G. E., and D. J. Henner. 1988. Multiple early transcripts and splicing of the *Autographa californica* nuclear polyhedrosis virus IE-1 gene. *J. Virol.* **62**:3193-3200.
- Clem, R. J., and L. K. Miller. Control of programmed cell death by the baculovirus genes *p35* and *iap*. *Mol. Cell. Biol.*, in press.
- Crawford, A. M., and L. K. Miller. 1988. Characterization of an early gene accelerating expression of late genes of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* **62**:2773-2781.
- Crook, N. E., R. J. Clem, and L. K. Miller. 1993. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol.* **67**:2168-2174.
- Friesen, P. D., and L. K. Miller. 1986. The regulation of baculovirus gene expression. *Curr. Top. Microbiol. Immunol.* **131**:31-50.
- Fuchs, L. Y., M. S. Woods, and R. F. Weaver. 1983. Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection: a novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. *J. Virol.* **43**:641-646.
- Glocker, B., R. R. Hoopes, Jr., L. Hodges, and G. F. Rohrmann. 1993. In vitro transcription from baculovirus late gene promoters: accurate mRNA initiation by nuclear extracts prepared from infected *Spodoptera frugiperda* cells. *J. Virol.* **67**:3771-3776.
- Gordon, J. D., and E. B. Carstens. 1984. Phenotypic characterization and physical mapping of a temperature sensitive mutant of *Autographa californica* nuclear polyhedrosis virus defective in DNA synthesis. *Virology* **138**:69-81.
- Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**:1044-1051.
- Grula, M. A., P. L. Buller, and R. F. Weaver. 1981. α -Amanitin-resistant viral RNA synthesis in nuclei isolated from nuclear polyhedrosis virus-infected *Heliothis zea* larvae and *Spodoptera frugiperda* cells. *J. Virol.* **38**:916-921.
- Guarino, L. A., and M. D. Summers. 1986. Functional mapping of a *trans*-activating gene required for expression of a baculovirus delayed-early gene. *J. Virol.* **57**:563-571.
- Guarino, L. A., and M. D. Summers. 1987. Nucleotide sequence and temporal expression of a baculovirus regulatory gene. *J. Virol.* **61**:2091-2099.
- Guarino, L. A., and M. D. Summers. 1988. Functional mapping of *Autographa californica* nuclear polyhedrosis virus genes required for late gene expression. *J. Virol.* **62**:463-471.
- Hoopes, R. R., and G. F. Rohrmann. 1991. In vitro transcription of baculovirus immediate early genes—accurate messenger RNA initiation by nuclear extracts from both insect and human cells. *Proc. Natl. Acad. Sci. USA* **88**:4513-4517.
- Huh, N. E., and R. F. Weaver. 1990. Categorizing some early and late transcripts directed by the *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol.* **71**:2195-2200.
- Huh, N. E., and R. F. Weaver. 1990. Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol.* **71**:195-202.
- Lee, H. H., and L. K. Miller. 1978. Isolation of genotypic variants of *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* **27**:754-767.
- Li, Y., A. L. Passarelli, and L. K. Miller. 1993. Identification, sequence, and transcriptional mapping of *lef-3*, a baculovirus gene involved in late and very late gene expression. *J. Virol.* **67**:5260-5268.
- McMaster, G. K., and G. G. Carmichael. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA* **74**:4835-4838.
- Miller, L. K., R. E. Trimarchi, D. Browne, and G. D. Pennock. 1983. A temperature sensitive mutant of the baculovirus *Autographa californica* nuclear polyhedrosis virus defective in an early function required for further gene expression. *Virology* **126**:376-380.
- Morris, T., and L. K. Miller. 1994. Mutational analysis of a baculovirus major late promoter. *Gene*, **140**:147-153.
- Ooi, B. G., C. Rankin, and L. K. Miller. 1989. Downstream sequences augment transcription from the essential initiation site of a baculovirus polyhedrin gene. *J. Mol. Biol.* **210**:721-736.
- O'Reilly, D. R., L. K. Miller, and V. A. Luckow. 1992. Baculovirus expression vectors: a laboratory manual. W. H. Freeman and Company, New York.
- O'Reilly, D. R., A. L. Passarelli, I. F. Goldman, and L. K. Miller. 1990. Characterization of the DA26 gene in a hypervariable region of the *Autographa californica* nuclear polyhedrosis virus genome. *J. Gen. Virol.* **71**:1029-1037.
- Partington, S., H. Yu, A. Lu, and E. B. Carstens. 1990. Isolation of temperature sensitive mutants of *Autographa californica* nuclear polyhedrosis virus phenotype characterization of baculovirus mutants defective in very late gene expression. *Virology* **175**:91-102.
- Passarelli, A. L., and L. K. Miller. 1993. Identification and characterization of *lef-1*, a baculovirus gene involved in late and very late gene expression. *J. Virol.* **67**:3481-3488.
- Passarelli, A. L., and L. K. Miller. 1993. Identification of genes encoding late expression factors located between 56.0 and 65.4 map units of the *Autographa californica* nuclear polyhedrosis virus genome. *Virology* **197**:704-714.
- Passarelli, A. L., and L. K. Miller. 1993. Three baculovirus genes involved in late and very late gene expression: *ie-1*, *ie-n*, and *lef-2*. *J. Virol.* **67**:2149-2158.
- Passarelli, A. L., and L. K. Miller. 1994. In vivo and in vitro analyses of recombinant baculoviruses lacking a functional *cg30* gene. *J. Virol.* **68**:1186-1190.
- Rankin, C., B. G. Ooi, and L. K. Miller. 1988. Eight base pairs encompassing the transcriptional start point are the major determinant for baculovirus polyhedrin gene expression. *Gene* **70**:39-50.

35. **Ribeiro, B., K. Hutchinson, and L. K. Miller.** 1994. A mutant baculovirus with a temperature-sensitive IE-1 transregulatory protein. *J. Virol.* **68**:1075–1084.
36. **Rice, W. C., and L. K. Miller.** 1986. Baculovirus transcription in the presence of inhibitors and in nonpermissive *Drosophila* cells. *Virus Res.* **6**:155–172.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. **Theilmann, D. A., and S. Stewart.** 1991. Identification and characterization of the IE-1 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. *Virology* **180**:492–508.
39. **Thiem, S. M., and L. K. Miller.** 1989. Identification, sequence, and transcriptional mapping of the major capsid protein gene of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* **63**:2008–2018.
40. **Thiem, S. M., and L. K. Miller.** 1989. A baculovirus gene with a novel transcription pattern encodes a polypeptide with a zinc finger and a leucine zipper. *J. Virol.* **63**:4489–4497.
41. **Thiem, S. M., and L. K. Miller.** 1990. Differential gene expression mediated by late, very late, and hybrid baculovirus promoters. *Gene* **91**:87–94.
42. **Tomalski, M. D., J. Wu, and L. K. Miller.** 1988. The location, sequence, transcription and regulation of a baculovirus DNA polymerase gene. *Virology* **167**:591–600.
43. **Vaughn, J. L., R. H. Goodwin, G. J. Tompkins, and P. McCawley.** 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *In Vitro* **13**:213–217.
44. **Weaver, R. F., and C. Weissman.** 1979. Mapping of RNA by a modification of the Berk-Sharp procedure the 5' termini of 15S beta-globin mRNA precursor and mature 10S beta-globin mRNA have identical map coordinates. *Nucleic Acids Res.* **7**:1175–1193.
45. **Wilson, M. E., T. H. Mainprize, P. D. Friesen, and L. K. Miller.** 1987. Location, transcription, and sequence of a baculovirus gene encoding a small arginine-rich polypeptide. *J. Virol.* **61**:661–666.