JOURNAL OF VIROLOGY, Feb. 1995, p. 968–974 Vol. 69, No. 2
0022-538X/95/$04.00+0

Eighteen Baculovirus Genes, Including *lef-11*, *p35*, *39K*, and *p47*, Support Late Gene Expression

JASON W. TODD,[1,2] A. LORENA PASSARELLI,[1] AND LOIS K. MILLER[1,2]*

Department of Genetics[1] and Department of Entomology,[2] The University of Georgia, Athens, Georgia 30602-2603

Received 1 August 1994/Accepted 28 October 1994

We report the identification of four additional genes of the *Autographa californica* nuclear polyhedrosis virus involved in expression from a late baculovirus promoter in transient expression assays. Three of these genes, *p35*, *39K*, and *p47*, have been previously described. The role of the *p35* gene product in late gene expression may be related to its ability to block apoptosis, since two other baculovirus genes also known to block apoptosis, Cp-*iap* and Op-*iap*, were able to functionally replace *p35* in the transient expression assay. The requirement for *p47* in this assay confirms its role in late gene expression, a role previously established by characterization of a temperature-sensitive mutant of *p47*, while the requirement for *39K* may be related to its known association with the virogenic stroma. The fourth gene identified as a late expression factor gene, *lef-11*, was located immediately upstream of *39K* and is predicted to encode a 13-kDa polypeptide. When plasmids containing these 4 genes were cotransfected with plasmids containing the 14 genes previously identified as late gene expression factors, the level of expression from the late capsid promoter was similar to that observed for a library of clones representing the entire viral genome. The genes provided by these 18 plasmids thus represent the viral genes necessary and sufficient to support expression from a late viral promoter in this transient expression assay.

Baculoviruses possess a large circular DNA genome which replicates in the nuclei of infected cells and is transcribed in three temporally distinct phases: early, late, and very late. The transition between baculovirus early and late gene transcription depends on DNA replication and involves a novel alpha-amanitin-resistant RNA polymerase (reviewed in references 3 and 24). As a step in understanding the nature of the novel polymerase activity and the mechanism by which DNA replication and transcription are coupled, we are identifying all of the viral genes required for late gene expression.

To identify these genes, we have employed a transient expression assay based on the ability of an overlapping library of clones, representing the entire genome of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV), to support expression of a reporter gene under late viral promoter control. By replacing individual or overlapping sets of clones from the library with plasmid subclones capable of supplying the transactivating activity, 14 Ac*M*NPV genes required for maximum expression in this assay have been identified (18, 21, 22, 26–29, 31). These genes are named late expression factor genes 1 to 10 (*lef-1* to *lef-10*), *ie-1*, *ie-2* (also referred to as *ie-n*), *dnapol*, and *p143*. Three of these genes, *dnapol*, *p143*, and *lef-8*, encode polypeptides with sequence motifs shared by DNA polymerases, DNA helicases, and RNA polymerases, respectively (19, 31, 37). The *ie-1* and *ie-2* genes are known to transactivate early genes (4, 14, 15), and they are involved in transactivating at least some of the *lef*s (14, 20, 22, 23).

In this study, we demonstrated the involvement of four additional Ac*M*NPV genes in late gene expression. The four genes were as follows: *lef-11*, a gene previously characterized by nucleotide sequence only; *39K*, a gene encoding a phosphoprotein (pp31) associated with the nuclear virogenic stroma (12); *p47*, a gene previously assigned a function in late and very late gene expression through the characterization of a conditional lethal mutant, *ts*317 (5, 25); and *p35*, a gene with known apoptosis suppressing activity in *Spodoptera frugiperda* cells (6, 7). Moreover, we provide evidence that a set of plasmids collectively encoding the 18 *lef*s and designated the lef library was sufficient to support late gene expression to levels similar to that observed for the entire overlapping genomic library.

* Corresponding author. Mailing address: Department of Entomology, University of Georgia, 413 Biological Sciences Bldg., Athens, GA 30602-2603. Phone: (706) 542-2294. Fax: (706) 542-2279. Electronic mail address: miller@bscr.uga.edu.

MATERIALS AND METHODS

Cells. The cell line IPLB-SF-21 (SF-21) (38), derived from the fall armyworm, *Spodoptera frugiperda*, was used for transfections. SF-21 cells were grown at 27°C in TC-100 medium (GIBCO BRL, Gaithersburg, Md.) containing 10% fetal bovine serum and 0.26% tryptose broth (24).

Transfections and transient expression assays. All transfections were performed by using calcium phosphate (24) to coprecipitate 2 μg of reporter plasmid, approximately 0.5 μg of any other DNA, and salmon sperm DNA to standardize the total concentration of DNA per reaction in each experiment (28).

Protein lysates were collected at 24, 48, and 72 h postcotransfection for samples containing pETCAThr5, pCAPCAT, and phcwt, respectively, and chloramphenicol acetyltransferase (CAT) assays (11, 33) were done by using 1/10 of the lysate or dilutions thereof for quantitation purposes.

Reporter plasmids. The previously described reporter plasmids pETCAThr5 (28), pCAPCAT (36), and phcwt (33) contain promoters of the early *etl* gene, the late major capsid protein gene, *vp39*, and the very late polyhedrin gene, *polh*, respectively, controlling the reporter gene *cat*. These reporter plasmids also contain hr5 sequences.

Plasmids. The plasmids pBCNE (26), containing *lef-1*; p630 (28), containing *lef-2*; pSDEM2 (18), containing *lef-3*; 86D5B (27), containing *lef-4*; pORF6 (27), containing *lef-5*; pAcIAP-Ps/NsiI (29), containing *lef-6* and a frameshift mutation within Ac-*iap*; pBSXBglII (22), containing *lef-7*; pRI-M (31), containing *lef-8*; pPstHI-SB2.35 (21), containing *lef-9*; and pPstHI-Sal0.8 (21), containing *lef-10*, have been previously described.

Other plasmids containing genes also involved in late gene expression, such as pIE1/HC (28), containing *ie-1*; pPstN (28), containing *ie-2*; pRI-D/SdORF5 (27), containing *p143*; and pDNAP (21), containing the DNA polymerase gene, *dnapol* (37), have also been previously described. pRS contains *p35* within the *Eco*RI-S fragment (86.8 to 87.9 map units [m.u.]) (10), pSB490 contains Cp-*iap* (9), and

p6-1 contains Op-*iap* (2). pAcIAP-Ps contains the Ac-*iap* and *lef-6* genes, while pAcIAP-PsdelHXdelSal contains Ac-*iap* and a deletion within *lef-6* (29).

A plasmid containing p47, pETL7/SB, was obtained by digesting the Ac*M*NPV library clone ETL7 with *Bst*EII, blunt ending it with the large fragment of DNA polymerase I (Klenow), and then digesting it with *Sst*I. A 4.2-kbp fragment was gel isolated and ligated to pBluescript SK+ (Stratagene, La Jolla, Calif.) previously digested with *Eco*RV and *Sst*I. p47 is a subclone of pETL7/SB generated by digesting the latter plasmid with *Xho*I and cloning a 2.4-kbp fragment containing Ac*M*NPV sequences from the *Xho*I site at 24.2 m.u. to the *Bst*EII site at 25.7 m.u. into the *Xho*I site of pBluescript SK+.

Plasmid pPstI-K contains the *Pst*I-K fragment (20.6 to 23.1 m.u.) cloned into the *Pst*I site of pBluescript SK. PstI-K includes the 39-kDa protein gene, *39K*, and open reading frame 5 (ORF 5) (*lef-11*) genes. Subclones of pPstI-K, pFspAfl, and pNspAfl, containing *39K*, were obtained by digesting pPstI-K with *Fsp*I and *Afl*II and with *Nsp*I and *Afl*II, respectively, blunt ending the resulting ends with T4 DNA polymerase, and cloning a 1.4- or 1.2-kbp fragment into the *Eco*RV site of pBluescript SK+. In addition to *39K*, pFspAfl contains the adjacent ORF 5 (13) and about 70 bp upstream of ORF 5, while pNspAfl contains only a portion of ORF 5.

pH_3R contains the *Hin*dIII-R fragment (22.0 to 23.4 m.u.) cloned into the *Hin*dIII site of pUC8. pH_3R/NH (22.0 to 23.0 m.u.) was obtained by digesting pH_3R with *Nru*I and *Hin*dIII, blunt ending the resulting ends with T4 DNA polymerase, and cloning a 1.3-kbp fragment into the *Eco*RV site of pBluescript SK+.

Plasmid p47/XbaI has a frameshift mutation within *p47* generated by partially digesting p47 with *Xba*I, repairing the protruding ends with T4 DNA polymerase, and ligating them. This mutation was confirmed by nucleotide sequence analysis, and it contains four additional nucleotides (CTAG) at the *Xba*I site (nucleotide 1762 in reference 5). To create a frameshift mutation within *39K*, pFspAfl was partially digested with *Hin*dIII, blunt ended with T4 DNA polymerase, and then ligated, adding four nucleotides (TAGC) at position 1445 in the nucleotide sequence reported by Guarino and Smith (13); this plasmid was named pFspAfl/fs. pH_3R/ORF5fs contains a mutation in ORF 5 generated by digesting pH_3R with *Bst*XI, blunt ending it with T4 DNA polymerase, and then ligating it, destroying the *Bst*XI site. In order to create a mutation in ORF 6 (13), pH_3R was grown in the *dam*-defective *Escherichia coli* strain GM33 to facilitate digestion with *Bcl*I. pH_3R/ORF6fs was constructed by partially digesting pH_3R with *Bcl*I, blunt ending it with T4 DNA polymerase, and then ligating it, destroying the *Bcl*I site; upon sequencing of this site, a 59-bp insertion of unknown origin was found at the former *Bcl*I site. pRSdel has a 310-bp deletion in the *p35* ORF contained within *Eco*RI-S (8), and pSB490Nco, a derivative of pSB490 (9), has a frameshift mutation at the *Nco*I site within Cp-*iap*. Plasmids constituting the lef library (see Results) are summarized in Table 1.

RESULTS

The involvement of *p47*, the 39-kDa protein gene (*39K*), and a gene upstream of *39K* in late gene expression. Three adjacent Ac*M*NPV clones from the library of overlapping genomic clones which supports expression from the late *vp39* promoter (28) potentially overlap the region of the Ac*M*NPV genome from 20.1 to 23.4 m.u. (Fig. 1A). These three clones are HL8 (starting between 7.35 and 13.1 m.u. and ending between 22.0 and 23.4 m.u.), HL5 (starting between 14.2 and 14.7 m.u. and ending between 23.4 and 29.0 m.u.), and ETL7 (starting between 20.1 and 22.0 m.u. and ending between 33.5 and 34.8 m.u.) (Fig. 1A). Three library clones, BC5, HL8, and HL5, each contain a copy of *lef-6* (29). So that *lef-6* would not be limiting, a plasmid containing *lef-6*, pAcIAP-Ps/NsiI (29), was added to all transfections in which the HL8 and HL5 library clones were omitted. Omission of HL8, HL5, and ETL7 from the Ac*M*NPV library, while providing *lef-6* to compensate for the lack of HL8 and HL5, resulted in reduced levels of *cat* expression from the reporter plasmid pCAPCAT, which contains the promoter of the major late capsid protein gene, *vp39* (Fig. 1C; compare lanes 1 and 2).

To test the late gene expression requirements provided by HL8, HL5, and ETL7, we substituted these Ac*M*NPV library clones with plasmid pPstI-K and/or plasmid pETL7/SB (Fig. 1A and C, lanes 3 through 5). Neither pPstI-K nor pETL7/SB was able to substitute individually for HL8, HL5, and ETL7 (Fig. 1C, lanes 3 and 4), but when added simultaneously, the two plasmids were able to reconstitute substantial levels of expression (Fig. 1C; compare lanes 1, 2, and 5). pPstI-K contains *39K* and several additional flanking ORFs, while pETL7/SB contains *p47*, an ORF encoding a predicted 43-kDa protein (5), 905 bp upstream of *p47*, and several ORFs upstream of the *39K* ORF (Fig. 1).

The product of *p47* is known to be involved in late gene expression through the characterization of a temperature-sensitive mutant, *ts*317, which exhibits impaired transcription and translation of late and very late genes (5, 25). Since *p47* is located within pETL7/SB, we tested directly whether *p47* was participating in expression from the *vp39* promoter. A smaller *p47*-containing clone called p47 (Fig. 1A) was used instead of pETL7/SB (in the presence of pPstI-K), and similar levels of CAT activity were obtained (Fig. 1C, lanes 5 and 6), although

TABLE 1. Genes and plasmids constituting the lef library

Gene	Clone name(s) (reference)	Size of product (kDa)	Function or homology of product	Temporal expression[a]	Genomic clone(s) containing *lef*[b]
lef-1	pBCNE (26)	31	LEF	E	BC5
ie-1	pIE1/HC (28)	67	*trans* activator	E, L	IE15
ie-n	pPstN (28)	47	*trans* activator	E	IE15
lef-2	p630 (28)	24	LEF		IE15
lef-3	pSDEM2 (18)	45	LEF	E	PstH5
p143	pR1D/SdelORF5 (27)	143	DNA replication (helicase motif)	E	HC10
lef-4	86D5B (27)	54	LEF		HC10
lef-5	pORF6 (27)	31	LEF		HC10
lef-6	pAcIAP-Ps/Nsi or pAcIAP-Ps (29)	20	LEF	E, L	BC5, HL8, HL5
lef-7	pBSXBglII (22)	27	LEF	E	XmaB
lef-8	pRI-M (31)	102	RNA polymerase motif		PstH4, ETL7
lef-9	pPstH1SB2.35 (21)	59	LEF		PstH4, PstH1, PstH5
lef-10	pPstH1Sal0.8 (21)	9	LEF		PstH4, PstH1, ETL7
dnapol	pDNAP (21)	114	DNA polymerase	E	PstH1, PstH5
lef-11	pH_3R or pH_3R/NH[c]	13	LEF		HL8, HL5, ETL7
p47	p47[c]	47	LEF	E	HL5, ETL7
39K	pNspAfl or pFspAfl[c]	31	LEF	E, L	HL8, HL5, ETL7
p35	pRS (10)	35	Blocks apoptosis	E, L	HK5

[a] E and L refer to transcription in early and late phases of infection, respectively.
[b] Ac*M*NPV genomic clones were previously described (28); see also Fig. 3A.
[c] Construction of plasmid(s) is described in Materials and Methods.

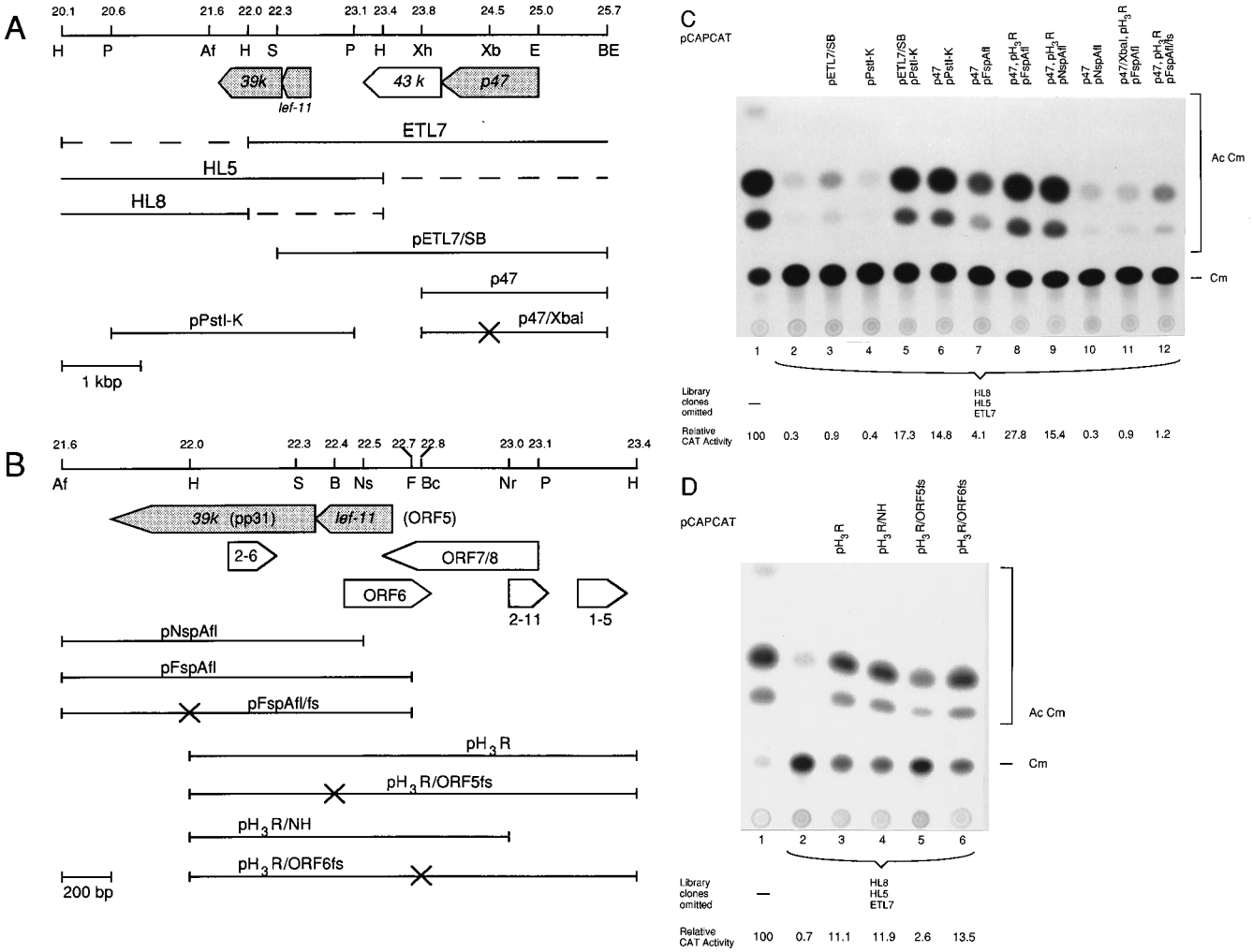

FIG. 1. Involvement of *p47*, *39K*, and a gene upstream of *39K* in late gene expression. (A) Restriction map of the Ac*M*NPV genome from 20.1 to 25.7 m.u. Previously characterized genes, *p47*, an ORF encoding a predicted 43-kDa protein (5), and *39K* (13) are shown as arrows below the map. The locations of relevant clones (ETL7, HL5, and HL8) as well as plasmid subclones (pETL7/SB, p47, and pPstI-K) are indicated. The broken parts of the lines indicating clones represent ambiguity about the ends of the genomic inserts of each clone. The frameshift mutation introduced into *p47* is shown as an X in the plasmid p47/XbaI. (B) Physical map of the region from 21.6 to 23.4 m.u. The locations and directions of ORFs larger than 48 amino acids are shown below the map. The *39K* ORF and ORF 5 (*lef-11*) are based on the sequence reported by Guarino and Smith (13), which was confirmed by Ayers et al. (1). ORFs 2-6, 2-11, and 1-5 are based on a sequence provided by Ayers et al. (1). This sequence contains an extra C at position 395 of the sequence reported by Guarino and Smith (13), which truncates ORF 6 by four amino acids and fuses ORFs 7 and 8 reported by Guarino and Smith, creating the single ORF indicated as ORF 7/8 in the diagram. We sequenced the region in question and confirmed that an extra C is also present in the L-1 variant. Plasmid clones used to map *lef-11* and to identify *39K* as a *lef* are shown relative to the expanded restriction map. Restriction sites shown in panels A and B are abbreviated as follows: H, *Hin*dIII; P, *Pst*I; S, *Sst*I; Xh, *Xho*I; Xb, *Xba*I; E, *Eco*RI; BE, *Bst*EII; Af, *Afl*II; B, *Bst*XI; Ns, *Nsp*I; F, *Fsp*I; Bc, *Bcl*I; Nr, *Nru*I. Not all the sites for the last six enzymes are shown. (C and D) Reporter plasmid pCAPCAT was cotransfected with the entire Ac*M*NPV library (lanes 1) or with the library lacking the clones HL8, HL5, and ETL7 (lanes 2 through 12 in panel C and lanes 2 through 6 in panel D). Plasmid pAcIAP-Ps/NsiI containing *lef-6* was included in lanes 2 through 12 of panel C and lanes 2 through 6 of panel D. Plasmid p47 was also included in lanes 2 through 6 of panel D, and pNspAfI was included in lanes 3 through 6 of panel D. For all figures showing data from CAT assays, additional plasmid subclones used in each cotransfection are shown above each lane and library clones included or omitted are indicated below each lane. The numbers under each lane indicate relative CAT activities of reactions as determined by PhosphorImager analysis (model 425E; Molecular Dynamics, Sunnyvale, Calif.) for dilutions of cell extracts so that the CAT assays were in the linear range of the assay (30% or less conversion of substrate to acetylated product). The data are representative of those from two or more similar experiments. The acetylated products (Ac Cm) and unacetylated substrate (Cm) are indicated on the right.

in neither case was full activity restored, possibly because the *p47* transcript normally terminates downstream of *39K* (Fig. 1A). This result and mutagenesis data shown below implicated *p47* as a late expression factor gene.

While providing p47 in the transfection mixtures, we then determined which subclones could replace pPstI-K. Addition of pFspAfI, a plasmid containing ORFs *39K* and ORF 5 and 75 additional nucleotides upstream of ORF 5 (Fig. 1A), did not reconstitute expression as well as addition of pPstI-K unless pH_3R (Fig. 1A) was also supplied (Fig. 1C, lanes 6 through 8). This indicated that pH_3R had additional transactivating activity; further evidence confirming this is shown in Fig. 1C (lanes 9 and 10). A combination of pNspAfI, a plasmid containing *39K* but lacking the upstream ORF 5 (Fig. 1A), and pH_3R supported levels of expression similar to those supported by pPstI-K (compare lanes 6 and 9), but when pH_3R was omitted (lane 10), only background levels of expression were obtained. The difference in levels of expression obtained when *39K* was supplied by either pFspAfI or pNspAfI (lanes 7 and 10) indicated that ORF 5 was involved in late gene expression but that

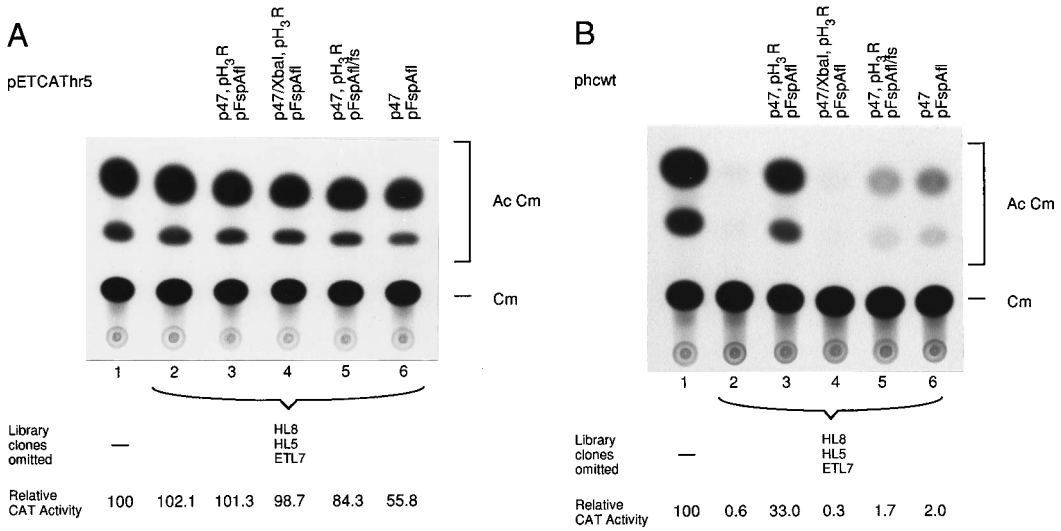

FIG. 2. Involvement of *p47*, *39K*, and *lef-11* in very late but not early gene expression. Reporter plasmids with an early promoter, pETCAThr5 (A), or a very late promoter, phcwt (B), were cotransfected with the entire Ac*M*NPV library (lanes 1) or with the library lacking the clones HL8, HL5, and ETL7 (lanes 2 through 6). Plasmid subclones added to each transfection are indicated above each lane. Lanes 2 through 6 also contain pAcIAP-Ps/NsiI to supply *lef-6*. The low level of expression in lanes 6 was probably due to inefficient expression of *lef-11* from pFspAf1. See the legend to Fig. 1C for additional details.

it may not have been effectively expressed from pFspAfI. On the basis of additional data shown below, we have renamed ORF 5 *lef-11*.

To confirm that *p47* was indeed involved in late gene expression, we created a frameshift mutation in *p47* and tested this plasmid, p47/XbaI, in our assay. Inactivation of *p47* in p47/XbaI reduced expression from the *vp39* promoter to background levels (Fig. 1C, lane 11), confirming the involvement of *p47* in late gene expression. At this time, we cannot rule out the possibility that the region upstream of *p47* in plasmid p47 also contains elements that are necessary for late gene expression.

Similarly, we showed that the product of *39K* was involved in late gene expression by creating a frameshift mutation in the ORF to make pFspAfI/fs and testing this plasmid with pH_3R in the assay. Truncation of the 39-kDa protein reduced late gene expression to the low level seen when pETL7/SB was added alone (Fig. 1C, lanes 3 and 12), indicating that *39K* was necessary for expression from the *vp39* promoter in this assay.

ORF 5 (*lef-11*) was the gene in pH_3R required for late gene expression. To demonstrate that ORF 5 was the *lef* present in pH_3R, we created a mutation at the *Bst*XI site within ORF 5 to make pH_3R/ORF5fs and compared the level of CAT activity produced by using this plasmid with that produced by using pH_3R. Alteration of ORF 5 significantly reduced activity (Fig. 1D; compare lanes 2, 3, and 5), confirming the involvement of ORF 5 (*lef-11*) in late gene expression. ORF 6, which overlaps with ORF 5, was not affected by the mutation in pH_3R/ORF5fs, but to confirm that ORF 6 was not enhancing expression, we also mutated ORF 6 within pH_3R (see Materials and Methods) and found that the transactivating activity of this plasmid (pH_3R/ORF6fs) was not reduced (Fig. 1D; compare lanes 3 and 6). Plasmid pH_3R/NH, which contains only the *Hin*dIII (22.0 m.u.)-to-*Nru*I (23.0 m.u.) region of pH_3R, was able to replace pH_3R (Fig. 1D; compare lanes 3 and 4), indicating that ORFs 7/8, 2-11, and 1-5 were not required for late gene expression.

***p47*, *39K*, and *lef-11* were required for very late but not early gene expression.** We tested the involvement of *p47*, *39K*, and *lef-11* in early and very late gene expression by using reporter plasmids pETCAThr5 and phcwt, respectively (Fig. 2). All three genes were necessary for expression from the *polh* promoter (Fig. 2B), but they did not appear to significantly affect expression from the early *etl* promoter (Fig. 2A).

***p35* stimulated expression from the *vp39* promoted-reporter gene.** Thus far, a total of 17 genes involved in late gene expression have been identified by using this transient expression assay (Fig. 3A and genes listed in Table 1, except *p35*). These genes include *lef-1* to *lef-11*, *ie-1*, *ie-2*, *p143*, *dnapol*, *p47*, and *39K* (18, 21, 22, 26–29, 31; this report). To determine if any additional genes were necessary for late gene expression, we began by omitting clones from the Ac*M*NPV library and replacing them with previously defined *lef*s so that there were progressively fewer library clones and more plasmid subclones. We first omitted three library clones, BC5, HL5, and HL8 (Fig. 3A), which we were certain could be fully complemented with plasmid clones containing *lef-1* and *lef-6* (pBCNE and pAcIAP-Ps, respectively) (29) (Fig. 3B, lanes 1 and 2). We were also able to complement IE15 effectively with *ie-1*, *ie-2*, and *lef-2* (pIE1/HC, pPstN, and p630, respectively) (28) when IE15, BC5, HL8, and HL5 were simultaneously removed from the library (Fig. 3B, lane 3). However, the additional omission of HK5 resulted in a noticeable reduction in gene expression (Fig. 3B, lanes 1 and 4). We had previously found that omission of HK5 in the context of the remaining Ac*M*NPV library results in an approximately threefold reduction of CAT activity from pCAPCAT (reference 26 and unpublished results).

We identified the gene in HK5 that stimulated late gene expression by using a set of 17 plasmids, each encoding a different *lef*, and adding HK5 or plasmid clones (Fig. 4A). Addition of HK5 increased expression to a level similar to that observed for the library (Fig. 4A, lanes 1 through 3). Plasmids pXJ, containing the *Xho*I-J fragment of Ac*M*NPV (data not shown), and pRS, a plasmid containing the *Eco*RI-S fragment, were each able to reconstitute expression to a level as high as or higher than that observed for the entire Ac*M*NPV library (Fig. 4A, lanes 1 and 4). The *Eco*RI-S fragment contains the apoptosis-inhibiting gene *p35* (6, 10) and a minor portion of the adjacent *p94* gene (10). To demonstrate that *p35* was the gene in HK5 that stimulated late gene expression, we used a plasmid containing a deletion within the ORF of *p35*, pRSdel.

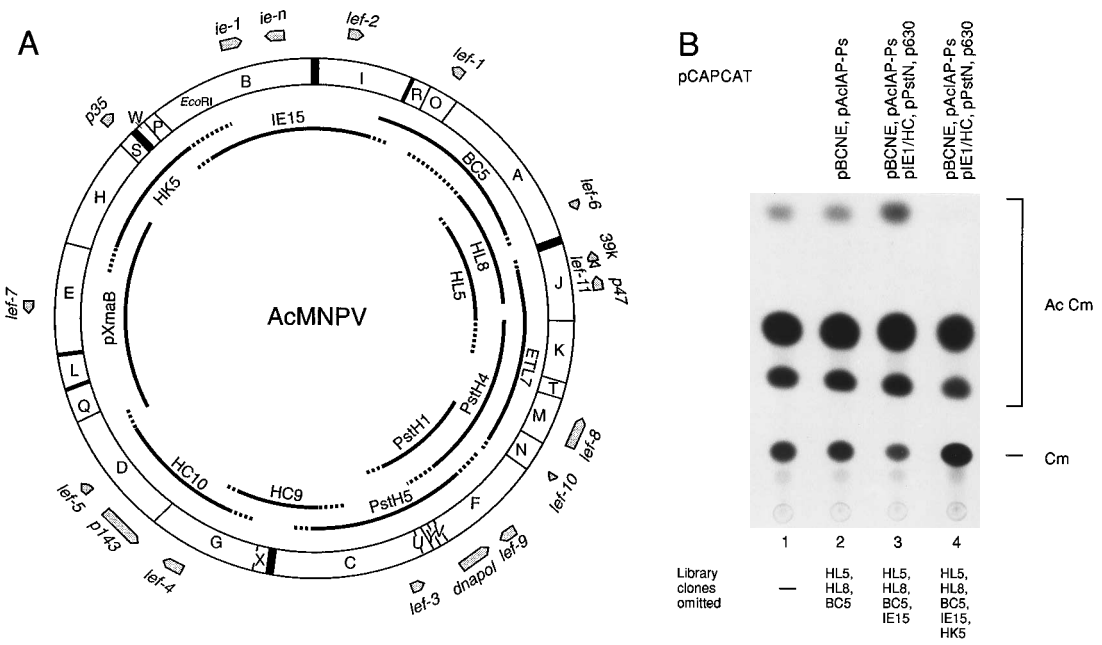

FIG. 3. Locations of Ac*M*NPV *lef*s and the effect of omitting the HK5 library clone on late gene expression. (A) Circular *Eco*RI map of Ac*M*NPV showing the locations of the overlapping Ac*M*NPV clones (bars inside map) and known *lef*s (arrows outside circle). The filled blocks within the *Eco*RI map indicate the locations of homologous regions. (B) Reporter plasmid pCAPCAT was cotransfected with the entire Ac*M*NPV library (lane 1) or with the library lacking the clones indicated under each lane (lanes 2 through 4). Plasmid subclones added to each transfection are indicated above each lane. The CAT activity was not quantitated in this experiment; the high levels of CAT resulted in a triacetylated product (top spot on chromatogram) which reflects the level of CAT activity but not quantitatively.

This plasmid did not increase expression above background levels (Fig. 4A; compare lanes 2 and 5). Thus, *p35* is involved in expression from the *vp39* promoter and provides an approximately fourfold stimulation of expression over that observed for the set of 17 *lef*s previously identified.

We were interested in determining if other baculovirus genes, Cp-*iap* and Op-*iap* (2, 9), which can substitute for *p35* in blocking apoptosis in SF-21 cells, could substitute for *p35* in this assay. Plasmids containing either Cp-*iap* (pSB490) or Op-*iap* (p6-1) were able to substitute for *p35*, while a plasmid carrying a frameshift mutation within Cp-*iap*, pSB490NcoI, was unable to substitute for *p35* or Cp-*iap* (Fig. 4A, lanes 6 and 7). Ac-*iap* is a homolog of Cp-*iap*, but it is unable to substitute for *p35* in blocking apoptosis (9); it was also unable to substitute for *p35* in enhancing *vp39* promoter activity, either with the downstream *lef-6* intact (lane 10) or with a deletion in *lef-6* (lane 9).

***p35* also stimulates very late but not early gene expression.** We tested the ability of *p35* to stimulate expression from the *etl* and *polh* promoters over levels supported by the set of 17 *lef*-containing plasmids. *p35* did not appear to enhance expression from the *etl* promoter (Fig. 4B, lanes 2 through 4), although early gene expression was monitored at 24 h after transfection and the effects of apoptosis may be less at this time. *p35* stimulated expression from the *polh* promoter about 10-fold compared with that observed for the set of previously identified *lef*s (Fig. 4C, lanes 2 through 4), but the 18 plasmids supported only about 10% of the level of expression supported by the Ac*M*NPV library (Fig. 4C, lanes 1 and 3), suggesting that the 18 *lef*s identified thus far were not sufficient for optimal *polh* expression.

DISCUSSION

We have demonstrated the involvement of 4 additional Ac*M*NPV genes in late and very late reporter gene expression in transient expression assays, bringing the total number of genes involved in late gene expression to 18. Three of the four genes reported here, *p47*, *39K*, and *lef-11*, were located in the region (20.1 to 25.7 m.u.) overlapped at least partially by three library clones, ETL7, HL5, and HL8. The fourth gene, *p35*, was found while we were determining which genes were necessary to achieve the same level of expression as that observed for the genomic clone library. A summary of the locations of the 18 genes is presented in Fig. 3A, and a list of the plasmids which support expression from the *vp39* promoter in transfected SF-21 cells is provided in Table 1.

The involvement of *p47* and *39K* in transient late gene expression was expected on the basis of prior characterization of these genes and/or their products (5, 12). The in vivo role of *p47* in late and very late gene expression, probably at the transcriptional level, was established through the characterization of the conditional lethal mutant *ts*317, which is predicted to have a single amino acid difference in the 47-kDa product (5, 25). The involvement of *39K* in late gene expression is likely to stem from the association of its phosphorylated gene product, pp31, with the virogenic stroma (12), a novel structure assembled in the nuclei of infected cells at late times (39).

We have also established a role for *lef-11* in late and very late gene expression. This gene, also known as ORF37 (1), lies immediately upstream of *39K* and, on the basis of its sequence (1, 13), is predicted to encode a highly basic (pI, 10.44) polypeptide of 13,130 daltons. A computer search of existing sequence databases revealed no obvious relationship to other genes except its counterpart in the *Orgyia pseudotsugata* nuclear polyhedrosis virus genome (34), which shares approximately 50% sequence identity with Ac*M*NPV *lef-11*. Our evidence that this ORF, rather than the overlapping ORF on the opposite strand, is responsible for the activation observed in our expression assay is based on the analysis of the effects of two mutations, one which left only the N-terminal two-thirds of

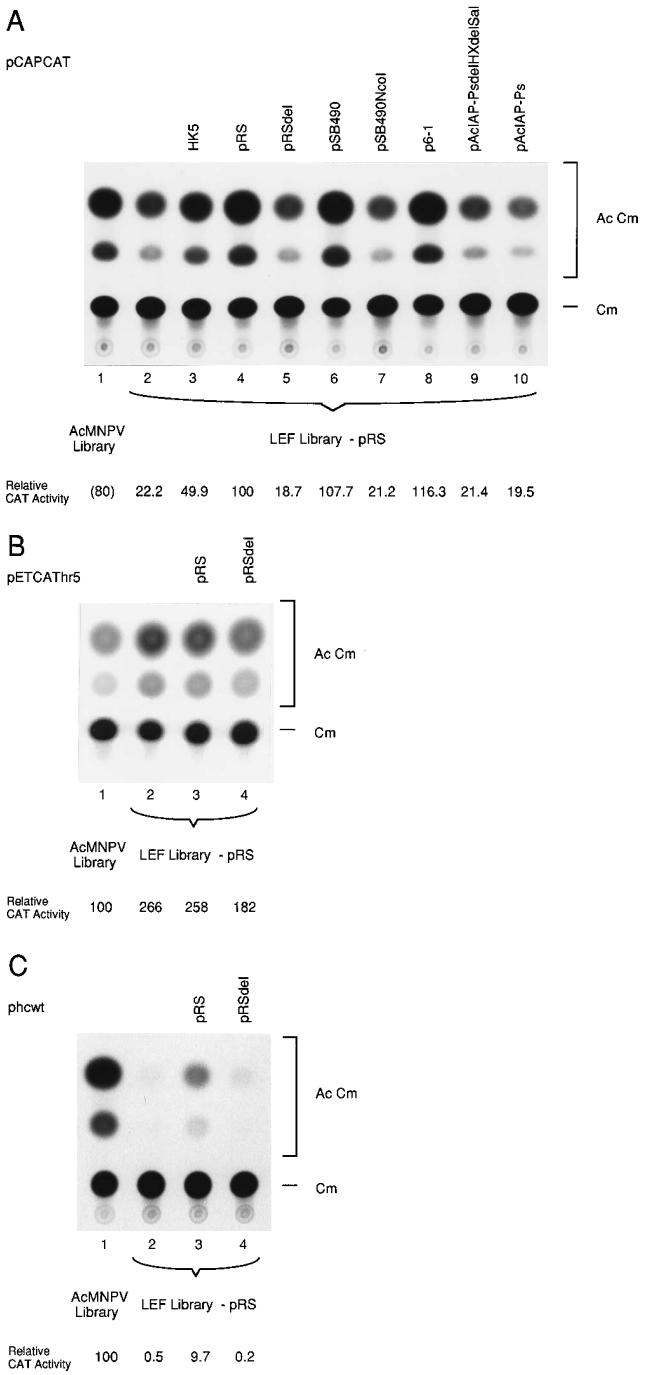

FIG. 4. A lef library including *p35* or *iap* genes supports transient gene expression. (A) *p35*, Cp-*iap*, and Op-*iap*, but not Ac-*iap*, stimulate expression from the late *vp39* promoter. Reporter plasmid pCAPCAT was cotransfected with the entire Ac*M*NPV library (lane 1) or with a set of 17 plasmids encoding *lef-1* to *lef-11* (pBCNE, p630, pSDEM2, 86D5B, pORF6, pAcIAP-Ps/NsiI, pBSXBglII, pRI-M, pPstHI-SB2.35, pPstHI-Sal0.8, and pH$_3$R, respectively), *ie-1*, *ie-2*, *p143*, *dnapol*, *p47*, and *39K* (pIE1/HC, pPstN, pRI-D/SdORF5, pDNAP, p47, and pFspAfl, respectively) (lanes 2 through 10). Additional plasmids or a library clone (HK5) was added as indicated above each lane to supply *p35* or an *iap*. The lef library including *p35* (supplied by pRS; lane 4) was used as the standard (100%) for relative CAT activities. The level of CAT activity for the Ac*M*NPV library was approximately 80% of this value. (B and C) *p35* enhances very late *polh* but not early *etl* promoter activity. Reporter plasmid pETCAThr5 (B) or phcwt (C) was cotransfected with the entire Ac*M*NPV library (lanes 1) or with the set of 17 plasmids described for panel A (lanes 2 through 4). Plasmids added to each transfection are indicated above each lane. See the legend to Fig. 1C for additional details.

ORF 6 but had no effect on reporter gene activity and a second one which is predicted to affect the translation of ORF 5 but not ORF 6 and which significantly decreased the level of reporter gene activity.

The presence of *p35* had a stimulatory effect on both late and very late gene expression in this assay. This gene is responsible for blocking apoptosis following Ac*M*NPV infection in SF-21 cells (6) and in other invertebrate and vertebrate cells (32, 35). The induction of apoptosis by Ac*M*NPV during the late phase results in reduced late and very late mRNA levels and impaired protein synthesis (7, 16). Because the effect of *p35* in our transient expression assays can be duplicated by either Cp-*iap* or Op-*iap*, two non-Ac*M*NPV genes which are able to complement *p35* and rescue *p35* mutants of Ac*M*NPV from apoptosis (2, 9), the role of *p35* in the assay appears to be at the level of blocking apoptosis in the transfected cells. This, in turn, suggests that expression of 1 of the plasmids, a subset of the plasmids, or all 18 plasmids induces apoptosis in SF-21 cells. However, we have not directly tested whether apoptosis is induced in the absence of *p35* in this transfection system, and the possibility remains that *p35* or *iap* genes also contribute more directly to late gene expression. This possibility is supported by the observation that higher levels of *p35* are required for normal levels of very late gene expression than are necessary to prevent apoptosis (17).

The definition of a set of viral genes involved in late gene expression is an important step in determining the mechanism by which transcription and DNA replication are coupled and the nature of the novel RNA polymerase involved in late gene transcription. The 18 genes we have defined by this assay support substantial levels of late gene expression, probably in conjunction with cellular factors, but additional viral genes are clearly required for optimal very late gene expression. Other viral genes (e.g., *pe-38* [30]) have additional stimulatory effects on late gene expression, but because of their subtler effects, we have not considered them central components of the late expression system. It is likely that only the 18 viral genes that we have defined contribute to the expression we observed, but we note that some of the genes required in the assay also contain internal ORFs which may contribute to expression. Knowledge of the genes involved will provide a direct approach for establishing in vitro systems for viral DNA replication and late gene transcription using extracts from uninfected cells supplemented with defined viral gene products. This information should also prove useful in defining host range determinants.

ACKNOWLEDGMENTS

J.W.T. and A.L.P. contributed equally to this work.

We thank Robert L. Possee (NERC Institute of Virology & Environmental Microbiology, Oxford, United Kingdom) for supplying the corrected version of the sequence of this region before publication. We also thank past and present members of the Miller laboratory for constructing some of the plasmids used in this study.

This work was supported in part by Public Health Service grant AI23719 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Ayers, M. D., S. C. Howard, J. Kuzio, M. Lopez-Ferber, and R. D. Possee.** The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology, in press.
2. **Birnbaum, M. J., R. J. Clem, and L. K. Miller.** 1994. An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. J. Virol. **68:**2521–2528.
3. **Blissard, G. W., and G. F. Rohrmann.** 1990. Baculovirus diversity and molecular biology. Annu. Rev. Entomol. **35:**127–155.
4. **Carson, D. D., L. A. Guarino, and M. D. Summers.** 1988. Functional mapping of an AcNPV immediately early gene which augments expression of the

ie-1 trans-activated 39k gene. Virology **162:**444–451.
5. **Carstens, E. B., A. L. Lu, and H. L. B. Chan.** 1993. Sequence, transcriptional mapping, and overexpression of p47, a baculovirus gene regulating late gene expression. J. Virol. **67:**2513–2520.
6. **Clem, R. J., M. Fechheimer, and L. K. Miller.** 1991. Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science **254:**1388–1390.
7. **Clem, R. J., and L. K. Miller.** 1993. Apoptosis reduces both the in vitro replication and the in vivo infectivity of a baculovirus. J. Virol. **67:**3730–3738.
8. **Clem, R. J., M. Robson, and L. K. Miller.** 1994. Influence of infection route on the infectivity of baculovirus mutants lacking the apoptosis-inhibiting gene *p35* and the adjacent gene *p94*. J. Virol. **68:**6759–6762.
9. **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.
10. **Friesen, P. D., and L. K. Miller.** 1987. Divergent transcription of early 35- and 94-kilodalton protein genes encoded by the *Hin*dIII K genome fragment of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J. Virol. **61:**2264–2272.
11. **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.
12. **Guarino, L. A., W. Dong, B. Xu, D. R. Broussard, R. W. Davis, and D. L. Jarvis.** 1992. Baculovirus phosphoprotein pp31 is associated with virogenic stroma. J. Virol. **66:**7113–7120.
13. **Guarino, L. A., and M. W. Smith.** 1990. Nucleotide sequence and characterization of the 39K gene region of *Autographa californica* nuclear polyhedrosis virus. Virology **179:**1–8.
14. **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.
15. **Guarino, L. A., and M. D. Summers.** 1987. Nucleotide sequence and temporal expression of a baculovirus regulatory gene. J. Virol. **61:**2091–2099.
16. **Hershberger, P. A., J. A. Dickson, and P. D. Friesen.** 1992. Site-specific mutagenesis of the 35-kilodalton protein gene encoded by *Autographa californica* nuclear polyhedrosis virus: cell line-specific effects on virus replication. J. Virol. **66:**5525–5533.
17. **Hershberger, P. A., D. L. LaCount, and P. D. Friesen.** 1994. The apoptotic suppressor P35 is required early during baculovirus replication and is targeted to the cytosol of infected cells. J. Virol. **68:**3467–3477.
18. **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.
19. **Lu, A., and E. B. Carstens.** 1991. Nucleotide sequence of a gene essential for viral DNA replication in the baculovirus *Autographa californica* nuclear polyhedrosis virus. Virology **181:**336–347.
20. **Lu, A., and E. B. Carstens.** 1993. Immediate-early baculovirus genes transactivate the p143 gene promoter of *Autographa californica* nuclear polyhedrosis virus. Virology **195:**710–718.
21. **Lu, A., and L. K. Miller.** 1994. Identification of three late expression factor genes within the 33.8- to 43.4-map-unit region of *Autographa californica* nuclear polyhedrosis virus. J. Virol. **68:**6710–6718.
22. **Morris, T. D., J. W. Todd, B. Fisher, and L. K. Miller.** 1994. Identification of lef-7: a baculovirus gene affecting late gene expression. Virology **200:**360–369.
23. **Nissen, M. S., and P. D. Friesen.** 1989. Molecular analysis of the transcriptional regulatory region of an early baculovirus gene. J. Virol. **63:**493–503.
24. **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.
25. **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.
26. **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.
27. **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.
28. **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.
29. **Passarelli, A. L., and L. K. Miller.** 1994. Identification and transcriptional regulation of the baculovirus *lef-6* gene. J. Virol. **68:**4458–4467.
30. **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.
31. **Passarelli, A. L., J. W. Todd, and L. K. Miller.** 1994. A baculovirus gene involved in late gene expression predicts a large polypeptide with a conserved motif of RNA polymerases. J. Virol. **68:**4673–4678.
32. **Rabizadeh, S., D. J. LaCount, P. D. Friesen, and D. E. Bredesen.** 1993. Expression of the baculovirus p35 gene inhibits mammalian neural cell death. J. Neurochem. **61:**2318–2321.
33. **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.
34. **Russell, R. L., and G. F. Rohrmann.** 1993. Nucleotide sequence of the ubiquitin-39K gene region from the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. J. Gen. Virol. **74:**1191–1195.
35. **Sugimoto, A., P. D. Friesen, and J. H. Rothman.** 1994. Baculovirus p35 prevents developmentally programmed cell death and rescues a ced-9 mutant in the nematode *Caenorhabditis elegans*. EMBO J. **13:**2023–2028.
36. **Thiem, S. M., and L. K. Miller.** 1990. Differential gene expression mediated by late, very late, and hybrid baculovirus promoters. Gene **91:**87–94.
37. **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.
38. **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 (Rockville) **13:**213–217.
39. **Young, J. C., E. A. MacKinnon, and P. Faulkner.** 1993. The architecture of the virogenic stroma in isolated nuclei of *Spodoptera frugiperda* cells in vitro infected by Autographa californica nuclear polyhedrosis virus. J. Struct. Biol. **110:**141–153.