JOURNAL OF VIROLOGY, Feb. 1995, p. 975–982 Vol. 69, No. 2
0022-538X/95/$04.00+0

The Roles of Eighteen Baculovirus Late Expression Factor Genes in Transcription and DNA Replication

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Received 1 August 1994/Accepted 28 October 1994

A set of 18 plasmid subclones of the *Autographa californica* nuclear polyhedrosis virus genome supports expression from a late viral promoter in transient expression assays (J. W. Todd, A. L. Passarelli, and L. K. Miller, J. Virol. 69:968–974, 1995). Using this set of plasmids, we have assigned a role for each of the 18 genes required for optimal late gene expression with respect to its involvement at the levels of transcription, translation, and/or DNA replication. RNase protection analyses demonstrated that all of the known late expression factor genes (*lef*s) affected the steady-state level of reporter gene RNA. Thus, none of the *lef*s appeared to be specifically involved in translation. A subset of the *lef*s supported plasmid replication; *ie-1*, *lef-1*, *lef-2*, *lef-3*, *p143*, and *p35* were essential for plasmid replication, while *ie-n*, *lef-7*, and *dnapol* had stimulatory effects. The predicted sequence of *lef-7* suggests that it is a homolog of herpesvirus single-stranded DNA-binding protein (UL29). The role of *p35* in plasmid replication appears to be suppression of apoptosis, because *p35* could be functionally replaced in the replication assay by either Cp-*iap* or Op-*iap*, two heterologous baculovirus genes which suppress apoptosis by a mechanism which appears to differ from that of *p35*. Thus, one or more of the replication-related *lef*s or the process of plasmid replication appears to induce cellular apoptosis. Our results indicate that the remaining *lef*s, *lef*s 4 through 11, *p47*, and *39K* (pp31), function either at the level of transcription or at that of mRNA stabilization.

The genome of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) consists of a circular double-stranded DNA molecule of 133,894 bp (1). During infection, Ac*M*NPV genes are expressed in a highly regulated cascade in which early gene expression and viral DNA replication are essential for late and very late gene expression (10, 13, 49). Early genes are transcribed by host RNA polymerase II (12, 21, 22), and they are transactivated by IE-1 and IE-2 (also known as IE-N), the products of *ie-1* and *ie-2*, respectively (17, 18, 26, 30, 33, 34, 38, 48). IE-1 is also required for expression from the late capsid protein gene (*vp39*) and very late polyhedrin gene (*polh*) promoters (38, 48), although this effect likely reflects the dependence of early gene expression on IE-1.

The transition between the early and late phases of gene expression is mediated by a switch from host RNA polymerase II to a novel alpha-amanitin-resistant RNA polymerase activity (15, 22). It is likely that this novel polymerase is encoded, at least in part, by the virus on the basis of biochemical evidence (11, 57) and the identification of two genes, *lef-8* and *lef-10*, encoding polypeptides with conserved RNA polymerase motifs (31, 43).

The dependence of late and very late gene expression on DNA replication was demonstrated by using inhibitors of DNA replication (49) and by characterizing a temperature-sensitive mutant of Ac*M*NPV, *ts*8, defective in DNA replication at the nonpermissive temperature (10, 13) and a null mutant of *et1*, a homolog of proliferating cell nuclear antigen (36), which exhibits a delay in late gene expression (8). The *ts*8 mutation is located within *p143*, a gene encoding a 143-kDa polypeptide with motifs conserved among DNA helicases (29). The dependence of late gene expression on DNA replication is also reflected by the involvement of both *p143* and the DNA polymerase gene, *dnapol*, in supporting expression of a reporter plasmid containing the chloramphenicol acetyltransferase (CAT) gene under the transcriptional control of the late *vp39* promoter (31, 40).

The DNA polymerase of *Orgyia pseudotsugata* nuclear polyhedrosis virus is required for origin-specific plasmid replication in a transient replication system (45) based on an assay whereby replicated plasmids are distinguished from transfected input plasmids by their resistance to cleavage by *Dpn*I (46). In this assay, specific regions of the Ac*M*NPV genome known as homologous repeat (hr) regions appear to act as origins of plasmid replication in Ac*M*NPV-infected cells (24, 27, 44) as well as in cells transfected with cosmid clones of Ac*M*NPV (25). The efficiency of hr-dependent plasmid replication is correlated with the number of palindromes present within each hr (44).

Eighteen late expression factor genes (*lef*s) are necessary to support optimal levels of transient expression from a reporter gene (CAT) under late *vp39* promoter control (28, 31, 33, 38–40, 42, 43, 54). These genes include *ie-1*, *ie-2*, *dnapol*, *p143*, and *lef-8*, described above. Information concerning the roles of three more of these genes, *39K* (pp31), *p47*, and *p35*, is available. The *39K* gene product is a phosphoprotein (pp31) associated with the virogenic stroma (15a), *p47* is involved in late and very late gene expression on the basis of characterization of a temperature-sensitive Ac*M*NPV mutant (3), and *p35* suppresses apoptosis in *Spodoptera frugiperda* (Sf) cells and larvae (5, 6) as well as in cells and larvae of other organisms (47, 52).

The roles of the remaining ten *lef*s have not been further defined, but they are likely to act at the level of transcription, translation, or viral DNA replication. To increase our understanding of the functions of each *lef*, we used a library consisting of 18 plasmids, each supplying one of the 18 *lef*s. This "lef" library is able to support late reporter gene expression to a level which is similar to that provided by a clone library over-

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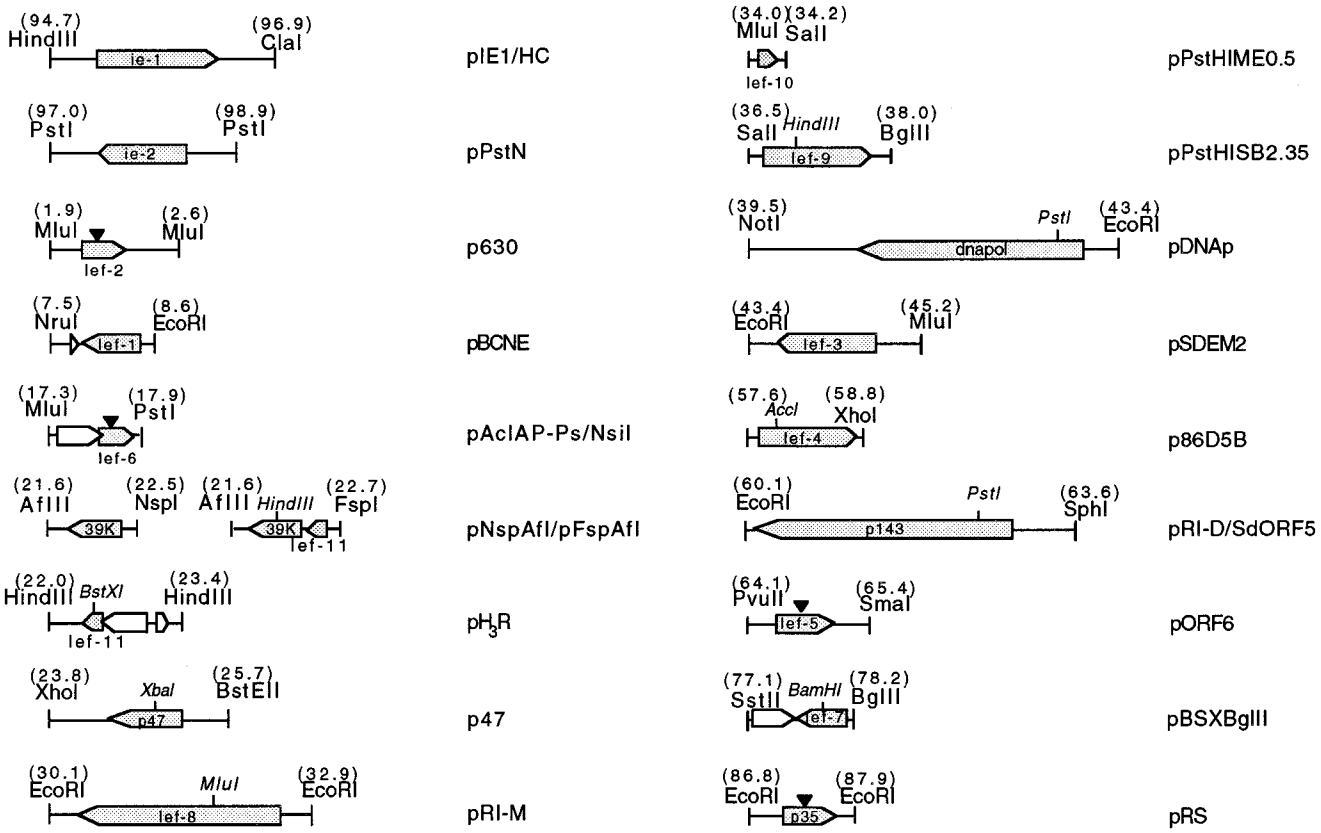

FIG. 1. Schematic representation of the plasmids composing the lef library. Arrowed boxes indicate the positions and orientations of complete nonoverlapping open reading frames (ORFs) within the Ac*M*NPV DNA insert of each plasmid. The indicated *lef* genes are represented by shaded boxes, and they correspond to the following ORFs designated by Ayres et al. (1) or to the exact nucleotide positions of the *lef* if the *lef* has not been assigned a number: *ie-1*/ORF147, *ie-2*/ORF151, *lef-2*/ORF6, *lef-1*/ORF14, *lef-6*/ORF26, *39K*/ORF36, *lef-11*/30401–30053, *p47*/ORF40, *lef-8*/*ORF50, lef-10*/45128–45364, *lef-9*/ORF62, *dnapol*/ORF65, *lef-3*/ORF67, *lef-4*/ORF90, *p143*/ORF95, *lef-5*/ORF99, *lef-7*/ORF125, and *p35*/ORF135. Map units and restriction sites corresponding to the limits of the viral insert are shown above each plasmid. Mutated versions of the *lef* genes are indicated by inverted black arrows (for deletions) or by italicized restriction site names (for frameshifts) corresponding to the sites at which the frameshift mutations were generated in the *lef* genes.

lapping the entire Ac*M*NPV genome. By removing one plasmid at a time, we determined whether they affected plasmid DNA replication or steady-state RNA levels. We found that all of the *lef*s affected the steady-state levels of reporter gene transcripts and that nine were required for or stimulated plasmid replication.

MATERIALS AND METHODS

Cells. SF-21 cells were cultured at 27°C in TC-100 medium (GIBCO BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum and 0.26% tryptose broth (37).

Plasmid constructs. The reporter plasmid used in all experiments, pCAPCAT (53), contains the CAT gene under the transcriptional control of the late *vp39* promoter (53). This construct also contains a 473-bp *Mlu*I fragment from the Ac*M*NPV *Hin*dIII-Q fragment containing four of six complete palindromes of hr5 (16), a *cis*-acting sequence which serves as an origin of Ac*M*NPV-activated replication in transient plasmid replication assays (27, 44).

The plasmids composing the lef library (Fig. 1) and their corresponding *lef*-specific mutations, including frameshifts (fs), deletions (del), or other inactivating mutations (mu), have been previously described as follows: pBCNE (*lef-1*) (39); p630 (*lef-2*) and p630-del (*lef-2* del), pIE1/HC (*ie-1*), and pPstN (*ie-n*) (38); pSDEM2 (*lef-3*) (28); p86D5B (*lef-4*) and p86D5BAccI (*lef-4* fs), pRI-D/SdORF5 (*p143*), and pORF6 (*lef-5*) and pORF6del (*lef-5* del) (40); pAcIAP-Ps/NsiI (*lef-6*) and pAcIAPdelHXdelSal (*lef-6* del) (42); pBSXBglII (*lef-7*) and pLEF7fs (*lef-7* fs) (33); pRI-M (*lef-8*) and pRI-M/MluI (*lef-8* fs) (43); pPst HISB2.35 (*lef-9*) and pPstHISB2.35fsHdIII (*lef-9* fs), pPstHIME0.5 (*lef-10*) and pPstHIME0.5fs (*lef-10* fs), and pDNAp (*dnapol*) and pDNApfs (*dnapol* fs) (31); pH_3R (*lef-11*) and pH_3Rfs (*lef-11* mu), pNspAfI (*39K*) and pFspAfI/fs (*39K* fs), p47 (*p47*) and p47/XbaI (*p47* fs), and pRS (*p35*) and pRSdel (*p35* del) (54).

The plasmid pUCp143 was constructed by digesting pRI-D/SdORF5 completely with *Eco*RI and partially with *Sph*I, isolating a 4,280-bp fragment, and cloning it into pUC19 digested with the corresponding restriction enzymes. pUCp143fsPstI was created by digesting pUCp143 with *Pst*I, blunt ending it with T4 DNA polymerase in the presence of deoxynucleoside triphosphates, and religating it. pPstI-K contains the *Pst*I-K fragment (20.6 to 23.1 map units) of Ac*M*NPV (54).

Plasmids pHSOpIAP, pHSCpIAP, and pHSAcIAP, containing the *Orgyia pseudotsugata* nuclear polyhedrosis virus, *Cydia pomonella* granulosis virus, and Ac*M*NPV *iap* genes under *hsp70* promoter control, respectively, are described elsewhere (7).

The overlapping Ac*M*NPV clone library used in these experiments has been described previously (38).

Transfection, RNA isolation, and RNase protection assays. SF-21 cells (2.0×10^6 cells per plate) were transfected by using lipofectin reagent (GIBCO BRL). Cells were transfected with 2.0 μg of the reporter plasmid, pCAPCAT, and either 0.5 μg of each of the clones of the overlapping Ac*M*NPV library or 1.0 μg of each plasmid of the lef library unless otherwise noted. Transfected cells were incubated at 27°C for 48 h, washed in TC-100 medium without fetal bovine serum, pelleted by low-speed centrifugation, and resuspended in 1,500 μl of TC-100 without serum. One-tenth of the cell suspension was removed for the preparation of cell lysates for CAT assays. The final volume of cell lysate prepared from these cells was 25 μl. CAT activity was determined with 5 μl of the lysate (14). Total cell RNA was isolated from the remainder of the cells by the guanidinium isothiocyanate-CsCl procedure (4). RNA concentrations were determined from the A_{260}.

RNA probes used in the RNase protection analyses were synthesized by using the Riboprobe II Core system (Promega Corp., Madison, Wis.) and either SP6 (Promega Corp) or T7 (GIBCO BRL) RNA polymerase. A 375-bp portion of the *S. frugiperda* glyceraldehyde phosphate dehydrogenase (GAPDH) gene corresponding to amino acid residues 195 to 310 of the *Drosophila melanogaster* GAPDH gene was used as a control for mRNA levels. This gene segment was isolated by using primers corresponding to conserved regions of GAPDH (5′-

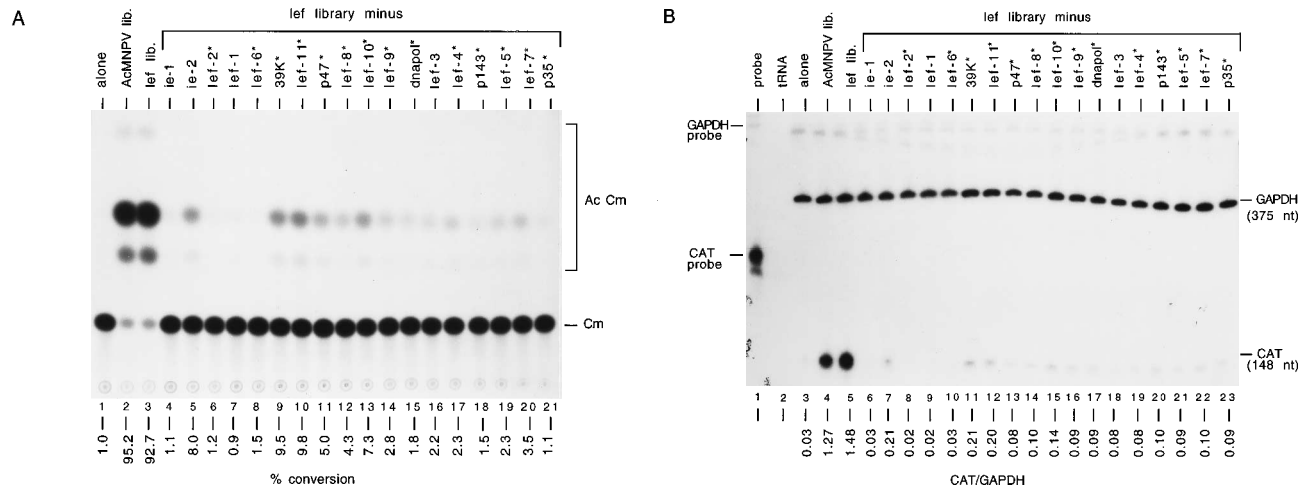

FIG. 2. *lef*s affect the steady-state levels of reporter gene (CAT) transcripts. (A) Transient expression assays showing the levels of CAT activity from SF-21 cells transfected with pCAPCAT alone (lane 1) or cotransfected with pCAPCAT and the complete Ac*M*NPV library of overlapping clones (lane 2), the lef library (lane 3), or the lef library lacking one of the eighteen *lef*s (indicated above each lane) (lanes 4 through 21). Those *lef* plasmids which were replaced by equivalent plasmids with *lef*-specific mutations are indicated by asterisks. The percent conversion of acetylated chloramphenicol is indicated below each lane. The positions of the acetylated (Ac Cm) and unacetylated forms (Cm) of chloramphenicol are indicated at the right. (B) RNase protection analysis of RNA isolated from SF-21 cells transfected as described above. The positions of unprotected GAPDH and CAT cRNA probes are shown in lane 1. The expected RNase-protected fragments for GAPDH (375 nt) and CAT (148 nt) are indicated on the right. Levels of protected CAT fragments for each transfection are given below each lane as a ratio of the amount of protected CAT fragments to the amount of protected GAPDH fragments. The results shown are representative of those from three independent experiments.

GACGGACCCTCTGGAAAACTG-3′ and 5′-ACCAGCTGATGAGCTTGAC GAA-3′) to obtain a PCR product which was cloned into pCR1000 (Invitrogen Corp., San Diego, Calif.). The resulting construct, pTASfGAPDH3′, was used to generate a cRNA probe to GAPDH by using T7 RNA polymerase in the presence of [^{32}P]UTP (3,000 Ci/mmol) following linearization of the template with *Eco*RI. The resulting cRNA probe was 458 nucleotides (nt) in length and contained 375 nt complementary to the *S. frugiperda* GAPDH transcript. Similarly a CAT-specific cRNA probe of 193 nt containing 148 nt complementary to the 5′ end of CAT mRNA was synthesized by linearizing p4CAT148 (51) with *Hin*dIII and transcribing it with SP6 RNA polymerase in the presence of [^{32}P]UTP (3,000 Ci/mmol). Transcription reactions were performed with 1 μg of template in a total of 20 μl according to the manufacturer's instructions. Templates were degraded by adding 5 U of RQ1 DNase (Promega Corp.) to the transcription reaction mixture and incubating it at 37°C for 1 h. Probes were separated from nonincorporated nucleotides by using Quick Spin (G-50 Sephadex) columns (Boehringer Mannheim, Indianapolis, Ind.).

RNase protection assays were performed as follows. Total cell RNA (100 μg) or tRNA (as an RNase digestion control) was coprecipitated in ethanol with 1.0×10^5 cpm of each cRNA probe, resuspended in 10 μl of TK buffer (550 mM Tris, pH 8.3, 450 mM KCl), and incubated at 85°C for 10 min. The hybridization temperature was reduced gradually to 42°C over a 1-h period, 300 μl of RNase digestion mix (40 μg of RNase A [Sigma, St. Louis, Mo.] per ml, 2 U of RNase T_1 [GIBCO BRL] per ml, 10 mM Tris [pH 7.5], 300 mM NaCl, 5 mM EDTA) was added, and the resulting mixture was incubated at 30°C for 1 h. RNase digestions were terminated by adding 20 μl of 10% sodium dodecyl sulfate and 10 μl of proteinase K (10 mg/ml) and incubating the mixture for 30 min at 37°C. Samples were phenol-chloroform and chloroform extracted, precipitated, and resuspended in 5 μl of loading mix (99% deionized formamide, 0.67% xylene cyanol, 0.033% bromophenol blue). Samples were heated at 95°C for 2 min and loaded onto a denaturing 5% polyacrylamide–8 M urea gel. After electrophoresis, autoradiography was performed by using Kodak film at −80°C. Quantitations of CAT assays and RNase protection assays were performed directly on the thin-layer chromatography plates and the gels, respectively, by using a Molecular Dynamics PhosphorImager 4000 (Sunnyvale, Calif.).

DNA replication assays. SF-21 cells (2.0×10^6 cells per plate) were cotransfected with pCAPCAT and clones of the overlapping Ac*M*NPV library or the lef library as detailed above. Total intracellular DNA was isolated at 96 h posttransfection as previously described (5). The DNA was precipitated and resuspended in 100 μl of TE (10 mM Tris, pH 8.0, 1 mM EDTA) (pH 8.0). One-tenth of the DNA was digested with 40 U of *Dpn*I and 40 U of *Bgl*II in a total volume of 40 μl at 37°C for 8 to 16 h, electrophoresed through a 0.7% agarose gel, and transferred to a Zeta-probe nylon membrane (Bio-Rad, Richmond, Calif.). Hybridizations were performed for 16 to 18 h by using a [^{32}P]dCTP nick-translated pCAPCAT probe with an approximate specific activity of 10^9 cpm/μg, and the membrane was washed under stringent conditions (50). Blots were exposed to X-ray film for 2 to 16 h at −80°C. The relative levels of plasmid DNA replication were determined by using the Molecular Dynamics PhosphorImager 4000.

RESULTS

Late expression factor gene products affect the steady-state levels of reporter gene transcripts. The lef library (54) consists of 18 plasmids which, when cotransfected into SF-21 cells, support levels of CAT expression from the late reporter plasmid pCAPCAT that are comparable to the levels observed when a complete overlapping clone library of the Ac*M*NPV genome is used (54) (Fig. 2A; compare lanes 2 and 3). Removal of any one of the *lef* plasmids from the lef library and substitution, when available, with a corresponding plasmid containing a mutation in that *lef* resulted in a significant decrease in CAT levels relative to those observed for the complete plasmid library (Fig. 2A; compare lane 3 with lanes 4 through 21). Relative levels of CAT activity were reduced between approximately 10-fold (in the cases of *ie-2*, *39K*, *lef-11*, and *lef-10*; Fig. 2A, lanes 5, 9, 10, and 13, respectively) and approximately 100-fold or more (in the cases of *ie-1*, *lef-2*, *lef-1*, and *lef-6*; Fig. 2A, lanes 4, 6, 7, and 8, respectively).

In order to determine whether the absence of these *lef*s affected transcription, we developed an RNase protection assay to assess the levels of reporter CAT transcripts in preparations of total cellular RNA isolated from the transfected cells. Two cRNA probes, one corresponding to a 375-nt segment of the *S. frugiperda* GAPDH cDNA (*gapdh*) and the other corresponding to 148 nt of CAT, the reporter gene, were used to measure the levels of these transcripts in transfected cells. Although we were initially uncertain of whether the levels of host *gapdh* RNA would serve as an adequate control for ensuring equal sampling of total cellular RNA, we have found with experience that the *gapdh* probe functions well in this regard, and very little variation in the levels of this RNA was found among well-prepared samples.

For each of the *lef*s examined by using this assay, there was a direct correlation between the amount of protected CAT probe (Fig. 2B) and the level of CAT activity. A CAT fragment of 148 nt, the expected size of the fully protected CAT segment, was observed for RNA isolated from cells transfected

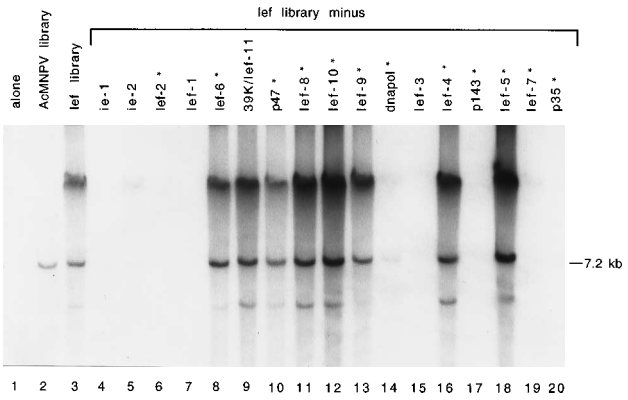

FIG. 3. Identification of *lef*s involved in plasmid DNA replication. Total intracellular DNA was isolated at 96 h posttransfection from SF-21 cells cotransfected with pCAPCAT alone (lane 1), pCAPCAT and the Ac*M*NPV overlapping clone library (lane 2), pCAPCAT and the lef library (lane 3), or pCAPCAT and the lef library in the absence of the indicated *lef*s (lanes 4 through 20). The position of the linear *Dpn*I-resistant 7.2-kb plasmid DNA fragment is indicated at the right of the panel. The lower band probably represents supercoiled plasmid, while the high-molecular-weight band may represent products of recombination between plasmids of the lef library and pCAPCAT. The results shown are representative of those from two independent experiments.

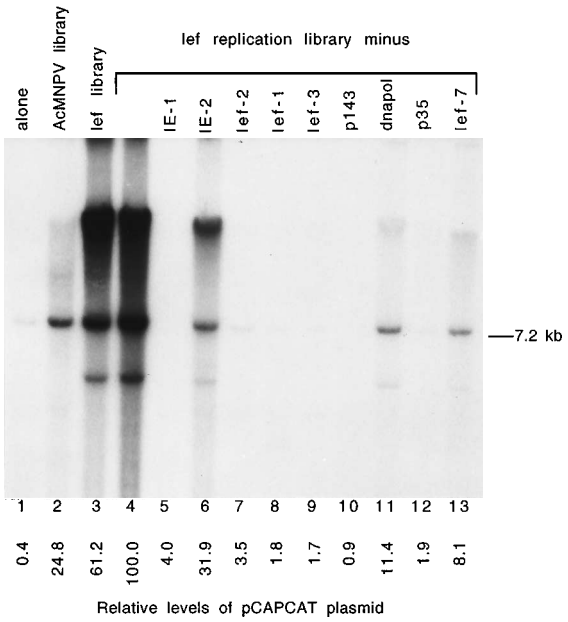

FIG. 4. Identification of *lef*s that stimulate plasmid DNA replication. SF-21 cells were cotransfected with pCAPCAT (lane 1), pCAPCAT and the complete Ac*M*NPV overlapping clone library (lane 2), pCAPCAT and the lef library (lane 3), pCAPCAT and the lef replication library (lane 4), or pCAPCAT and the lef replication library lacking one of the nine *lef*s involved in plasmid replication. The position of the linear *Dpn*I-resistant plasmid DNA fragment is indicated on the right. Levels of replicated plasmid were quantitated relative to those levels observed in the presence of the lef replication library (lane 4), and they are shown below each lane. The results shown are representative of those from two independent experiments.

with pCAPCAT and either the complete overlapping Ac*M*-NPV library or the lef library (Fig. 2B, lanes 4 and 5). No protected CAT probe was detected in cells transfected with pCAPCAT alone (Fig. 2B, lane 3). Omission or replacement of each *lef* from the lef library with a deleted or frameshifted version of the gene resulted in decreased to undetectable levels of protected CAT probe (Fig. 2B, lanes 6 through 23). In all cases the levels of protected GAPDH probe (375 nt) remained relatively constant. This indicated that the *lef*s primarily affected the levels of reporter gene transcripts rather than the translation of mRNA.

Nine late expression factor genes are involved in plasmid replication. To determine whether any of the *lef*s were affecting the level of reporter gene RNA by affecting reporter plasmid DNA replication, we determined whether our reporter plasmid, pCAPCAT, was replicated in the transient assay and which *lef*s were required for this replication. SF-21 cells were cotransfected with pCAPCAT and the Ac*M*NPV overlapping library, the lef library, or the lef library lacking one of the *lef* plasmids. Total intracellular DNA was prepared at 96 h posttransfection and digested with *Bgl*II to linearize pCAPCAT (7.2 kb) and *Dpn*I to distinguish between input plasmid DNA and replicated plasmid DNA (Fig. 3). No plasmid replication was detected in cells transfected with pCAPCAT alone (Fig. 3, lane 1); however, plasmid replication was observed in the presence of both the Ac*M*NPV overlapping library and the lef library (Fig. 3, lanes 2 and 3), indicating that the lef library was able to support plasmid DNA replication as well as or better than the Ac*M*NPV overlapping library.

By omitting one *lef* plasmid at a time from the lef library, we determined which *lef*s were required for or involved in plasmid DNA replication. Omission of plasmids containing *ie-1*, *ie-2*, *lef-2*, *lef-1*, *dnapol*, *lef-3*, *p143*, *lef-7*, and *p35* and substitution, when available, with their deleted or frameshifted counterparts resulted in either undetectable levels of plasmid replication or greatly reduced levels of plasmid replication. A longer exposure of the gel shown in Fig. 3 revealed that in the cases of *ie-2*, *dnapol*, and *lef-7*, some plasmid replication was occurring (exposure not shown; also see Fig. 4). No significant effects on plasmid replication were observed with the other *lef*s.

The high-molecular-weight fragments hybridizing to the pCAPCAT probe in lanes in which plasmid replication was detected may have been nicked circular pCAPCAT owing to incomplete *Bgl*II digestion. However, these fragments were larger than 12 kbp, so that it is unlikely that this was the case. A second possibility is that they were concatemers of pCAPCAT. The concatemerization of reporter plasmids in this assay was observed previously (27, 45). We do not believe this to be the case, since ladders corresponding to multiple-unit-length fragments were not observed if *Bgl*II was only partially digesting these concatemers. A third possibility is that we have been observing coreplication of the lef plasmid with pCAPCAT plasmids which were hybridizing with the probe via common vector sequences. However, this does not explain why we observed this high-molecular-weight DNA rather than DNA corresponding in size to each lef plasmid. A fourth possibility is that these high-molecular-weight fragments were the result of recombination within the common vector sequences of the lef plasmids and pCAPCAT. Most of these plasmids do not contain a *Bgl*II site, and consequently they would only be linearized as a result of the *Bgl*II site within pCAPCAT. We currently favor this hypothesis, since these high-molecular-weight fragments were not observed in the presence of the overlapping Ac*M*NPV library (Fig. 3 through 5).

Six of the nine *lef*s are required for plasmid DNA replication. Although the subtraction assays described above define those *lef*s involved in plasmid replication, it was necessary to confirm that together these *lef*s were both necessary and sufficient for plasmid replication. Figure 4 demonstrates that cells cotransfected with pCAPCAT and these nine *lef*s were able to support plasmid replication; in fact, the replication-specific *lef*s

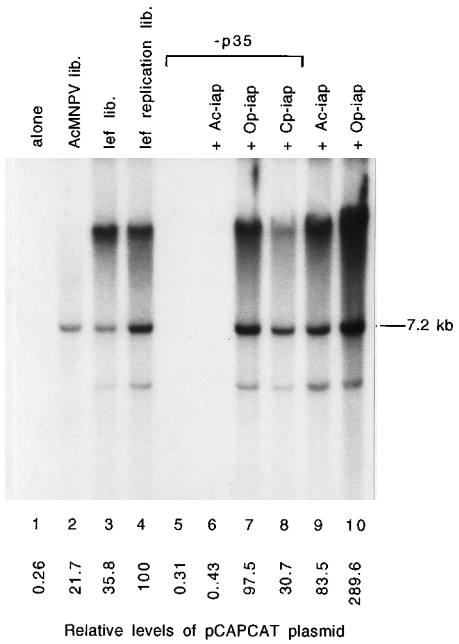

FIG. 5. Cp-*iap* and Op-*iap* can functionally substitute for *p35* in the plasmid replication assay. *Dpn*I assays were performed on cells cotransfected with pCAPCAT (lane 1), pCAPCAT and the Ac*M*NPV overlapping library (lane 2), pCAPCAT and the lef library (lane 3), pCAPCAT and the lef replication library (lane 4), pCAPCAT in the absence of *p35* (lane 5), and pCAPCAT and the lef replication library with *p35* substituted with Ac-*iap* (lane 6), Op-*iap* (lane 7), or Cp-*iap* (lane 8). In addition, the effects of Ac-*iap* and Op-*iap* in the presence of the lef replication library (including *p35*) are also shown (lanes 9 and 10, respectively). The position of the linear *Dpn*I-resistant plasmid DNA fragment is indicated on the right. The results shown are representative of those from two independent experiments.

supported higher levels of replication than either the complete lef library or the Ac*M*NPV overlapping clone library (Fig. 4; compare lane 4 with lanes 2 and 3). These nine *lef*s will be referred to as the lef replication library. Omission of *ie-1*, *lef-2*, *lef-1*, *lef-3*, *p143*, or *p35* from the lef replication library dramatically reduced the level of plasmid replication 25- to 100-fold (Fig. 4, lanes 5, 7 through 10, and 12), whereas omission of *ie-2*, *lef-7*, or, surprisingly, *dnapol* reduced plasmid replication only 3- to 12-fold (Fig. 4, lanes 6, 11, and 13). On the basis of these results, we consider IE-1, LEF-2, LEF-1, LEF-3, P143, and P35 required factors and IE-2, LEF-7, and DNA polymerase auxiliary factors for plasmid DNA replication in our assay system.

The inhibitor of apoptosis (IAP) genes of *Orgyia pseudotsugata* nuclear polyhedrosis virus and *Cydia pomonella* granulosis virus can functionally substitute for *p35* in plasmid replication. *p35* has been previously identified as an apoptotic suppressor gene, since SF-21 cells infected with Ac*M*NPV mutants lacking a functional *p35* gene undergo apoptosis (5, 19). Two genes, Cp-*iap* and Op-*iap*, which are able to suppress apoptosis in p35$^-$ mutant-infected SF-21 cells; have been reported to be present in other baculoviruses, *Cydia pomonella* granulosis virus (Cp-*iap*) and *Orgyia pseudotsugata* nuclear polyhedrosis virus (Op-*iap*); these genes are referred to as inhibitor of apoptosis (*iap*) genes. A homolog of the *iap*s, Ac-*iap*, is found in the Ac*M*NPV genome, but it is unable to suppress apoptosis in SF-21 cells (2, 9).

To determine whether any of these *iap*s could functionally replace *p35* in this plasmid replication assay, the effect of adding Ac-*iap*, Op-*iap*, or Cp-*iap* in place of *p35* on plasmid replication was tested. SF-21 cells were cotransfected with the lef replication library (Fig. 5, lane 4), the lef replication library with *p35* omitted, or the lef replication library with *p35* replaced by an *iap* homolog (Fig. 5, lanes 5 to 8). Omission of *p35* from the minimal plasmid library resulted in undetectable (background) levels of plasmid replication (Fig. 5; compare lanes 1 and 5). When *p35* was replaced by Ac-*iap*, no plasmid replication was observed, whereas addition of Op-*iap* and Cp-*iap* resulted in levels of plasmid replication comparable to and threefold lower than that observed for the lef replication library, respectively (Fig. 5; compare lanes 4, 7, and 8). This demonstrated that the effects exerted by the *iap* homologs and *p35* on plasmid DNA replication were likely related to their ability to block apoptosis in SF-21 cells.

A threefold increase in the level of plasmid replication was observed when Op-*iap* was added to the lef replication library (Fig. 5, lane 10), suggesting that the effect of Op-*iap* and *p35* on plasmid replication was additive or possibly synergistic. This effect was not observed when Ac-*iap* was added to the lef replication library (Fig. 6, lane 9). Increased levels of plasmid DNA replication were also observed when *p35* was added to the library in increasing quantities (data not shown), suggesting that this is a dosage effect of *p35*.

DISCUSSION

A subset of the 18 *lef* genes (*ie-1*, *ie-n*, *lef-1*, *lef-2*, *lef-3*, *dnapol*, *p143*, *lef-7*, and *p35*) were found to be involved in plasmid DNA replication. The finding that two viral transactivators, IE-1 and IE-2, are included in this subset is not surprising, since the expression of at least four of the above-described genes is upregulated by these transactivators (30, 33–35). The roles of the remaining replication-specific *lef*s in gene expression may be to provide sufficient template for optimal transcription and/or to couple late gene transcription to replication.

Six of these *lef*s, *ie-1*, *lef-1*, *lef-2*, *lef-3*, *p143*, and *p35*, were essential for plasmid DNA replication, while *ie-2*, *lef-7*, and *dnapol* stimulated plasmid DNA replication. While most of the genes we have found to be involved in plasmid DNA replication were the same as those described by Kool (23), differences were found in (i) the relative contributions of *dnapol* and *p35* to plasmid replication, (ii) the involvement of *pe-38* in the assay, and (iii) the involvement of *lef-7*.

In our assay, *dnapol* stimulated plasmid replication and *p35* was required for plasmid DNA replication, whereas Kool found that *dnapol* was essential and *p35* was stimulatory. The differences between these results may reflect differences in the reporter plasmid used or subleties in the technical approach, such as the time at which total intracellular DNA was harvested posttransfection for analysis. Kool used DNA isolated at 72 h posttransfection, whereas we have used DNA isolated at 96 h posttransfection. By 96 h posttransfection, extensive apoptosis and DNA nicking may occur in the absence of p35, so that a greater dependence on p35 is observed at 96 h posttransfection. Similarly, the absence of *dnapol* may delay plasmid DNA replication, so that its effects appear to be only stimulatory by 96 h posttransfection. Mutagenesis of *dnapol* within the viral genome will be necessary to determine if *dnapol* is essential for Ac*M*NPV DNA replication during infection.

Another difference between our lef replication library and the replication-specific genes identified by Kool was the role of *pe-38* in plasmid replication. Kool found that *pe-38* had a moderate effect on plasmid replication probably due to its ability to modestly stimulate expression from *p143* (30) and potentially other replication-specific *lef*s. The requirement for

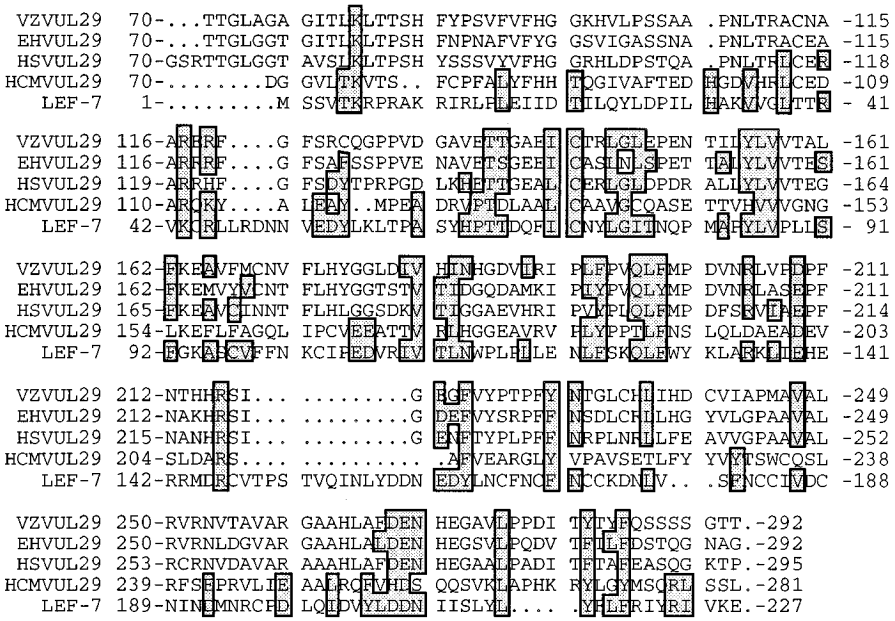

FIG. 6. Sequence alignments between *lef-7* and UL29. Alignments between the products of *lef-7* and UL29 from herpes simplex virus (HSVUL29) and UL29 homologs from varicella-zoster virus (VZVUL29), equine herpesvirus (EHVUL29), and human cytomegalovirus (HCMVUL29) are shown. The numbers correspond to the amino acid numbers relative to the translational start methionine. Similar (I/L, K/R, S/T, D/E, and F/Y/W) or identical amino acid residues between *lef-7* and the corresponding herpesvirus genes are boxed. Sequence alignments were performed by using the PILE-UP program from the Genetics Computer Group, University of Wisconsin.

pe-38 in plasmid replication was not tested in this study because *pe-38* was found to have only a modest (twofold) stimulatory effect on late (and very late) gene expression (38, 41) and consequently was not included in the lef library. We have recently tested the effect that *pe-38* has in our replication assay, and we have found that it has only a two- to threefold effect (data not shown).

The major difference between those replication-related genes identified by Kool (23) and our lef replication library was the role of *lef-7* in plasmid DNA replication. This gene was not identified by Kool (23), but it has the largest stimulatory effect (greater than 12-fold) on plasmid replication in our assay compared with *ie-2* and *dnapol*. It is possible that Kool did not identify *lef-7* as a replication-specific gene because we are using a different reporter plasmid and *lef-7* may be exerting an origin-specific effect; we have found that replication of pCAPCAT does not depend on the hr5 region and that pCAPCAT apparently contains an additional region which can serve as an origin of replication (32). It is also possible that *lef-7* was missed by Kool because it is located near a region exerting an inhibitory effect (33).

Extensive genetic analysis have identified seven herpes simplex virus type 1 genes (UL5, UL8, UL9, UL29, UL30, UL42, and UL52) that are necessary and sufficient for *ori*-specific plasmid replication in transfected cells (reviewed in reference 56). A database search with the LEF-7 sequence revealed significant sequence similarity at the amino acid level (21% identities over 227 amino acids) to the herpes simplex virus type 1 UL29 gene product (Fig. 6). In addition, a single-stranded DNA-binding sequence motif, $K/Rx_{2-5}K/Rx_{4-12}F/Yx_{2-14}F/Yx_{6-13}F/Yx_{1-19}K/Rx_{3-26}F/Y/Wx_{6-11}R/K$, determined to be present in all single-stranded DNA binding proteins from prokaryotic and eukaryotic organisms (55) is repeated twice within LEF-7. We, therefore, propose that *lef-7* encodes a single-stranded DNA binding protein.

In our assay, *p35* also appears to be required for plasmid replication, but this effect may be an indirect effect of the ability of *p35* to block apoptosis (5). On the basis of studies with *p35* mutants and inhibitors of macromolecular synthesis, Clem and Miller (7) proposed that either viral DNA replication or the subsequent shutoff of host RNA synthesis serves as a trigger for apoptosis. The requirement for *p35* in our transient replication system indicates that one or more of the replication *lef*s or possibly the process of plasmid DNA replication itself is able to induce apoptosis; late gene expression is apparently not required for the induction of apoptosis. We favor the view that DNA replication, which may appear as DNA damage to the cell, may trigger apoptosis, since aphidicolin blocks $p35^-$ mutant induction of apoptosis but not early gene expression.

The ability of Op-*iap* and Cp-*iap*, two genes that are also able to inhibit apoptosis by a mechanism which probably differs from that of *p35* (2, 7, 9), to substitute for *p35* in both the replication and expression assays supports the view that the role of *p35* in plasmid replication is an indirect one. The recent finding that p35 is located in the cytosol rather than the nucleus is another indicator that p35's effect on plasmid replication is indirect (20). However, we have not determined whether apoptosis is induced in the replication assay in the absence of p35 or IAP. It may be that p35 simply prevents the activation of the endonuclease associated with apoptosis.

All eighteen *lef*s affected the steady-state levels of reporter gene RNA. There was a strong correlation between the levels of CAT activity and the levels of CAT transcripts when individual *lef*s were omitted from the lef library. Thus, none of these *lef*s act exclusively or even strongly at the translational level. The remaining *lef*s which are not involved in plasmid DNA replication (*lef-4*, *lef-5*, *lef-6*, *lef-8*, *lef-9*, *lef-10*, *lef-11*, *39K*, and *p47*) are likely to function in late promoter recognition or stabilization of late transcripts or to participate directly in

transcription as subunits of the virus-induced RNA polymerase complex.

A comparison of the predicted molecular weights of these LEFs with the putative subunit composition of the virus-induced RNA polymerase purified by Yang et al. (57) suggests a correlation between the two. The identification of conserved RNA polymerase motifs found within the two largest subunits of prokaryotic and eukaryotic RNA polymerases which are also found within two LEFs, LEF-8 (43) and LEF-10 (31), supports the possibility that some of these *lef*s are components of the multisubunit Ac*M*NPV RNA polymerase.

The identification of those *lef*s involved in late and very late transcription and those *lef*s involved in plasmid DNA replication will facilitate a better understanding of the precise roles of each *lef* during baculovirus infection.

ACKNOWLEDGMENTS

We gratefully acknowledge the contributions of Elena Prikhodko, who helped construct pUCp143 and the corresponding frameshifted version, and Timothy Morris, who constructed pTASfGAPDH3′. We also thank Gary F. Merrill (Oregon State University) for providing the plasmid p4CAT148.

This work was supported in part by a Medical Research Council of Canada postdoctoral fellowship to A.L. and by Public Health Service grant AI23719 from the National Institutes of Allergy and Infectious Diseases to L.K.M.

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