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Nineteen Baculovirus Open Reading Frames, Including LEF-12, Support Late Gene Expression

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A set of 18 plasmid subclones of the *Autographa californica* nuclear polyhedrosis virus genome, each containing an identified late expression factor gene (*lef*), supports expression from a late viral promoter in transient expression assays in the SF-21 cell line derived from *Spodoptera frugiperda*. We have constructed a further set of plasmids in which each *lef* open reading frame (ORF) is controlled by the *Drosophila melanogaster* heat shock protein 70 (hsp70) promoter and epitope tagged. Failure of this set of plasmids to support transient late gene expression, and the inability of the *p47* ORF to replace the *p47*-containing plasmid supplied in the *lef* plasmid library, led to the identification of a 19th late expression factor gene (*lef-12*) located adjacent to the *p47* gene. The sequence of *lef-12* is predicted to encode a protein of 21 kDa with no homology to any previously identified protein. The set of 19 hsp70-controlled *lef* ORFs (HSEpiHis *lef* library) supports transient expression from a late viral promoter. *lef-12* did not affect expression from an early baculovirus promoter. In TN-368 cells, which are also permissive for virus replication, *lef-12* provided a stimulatory effect but did not appear to be essential.

Genes involved in regulating late gene expression of the baculovirus *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) have been identified primarily by using a transient expression assay which is based on activation of a reporter gene under control of a late viral promoter by cotransfection with a combination of viral genes (38, 41). In addition to the reporter gene, the reporter plasmid used in this assay contains an Ac*M*NPV homologous repeat (*hr*) region which serves as an origin of DNA replication (21, 22, 28, 43). When the reporter plasmid is cotransfected into cells in the presence of 12 overlapping clones (designated the genomic library) which collectively represent the Ac*M*NPV genome, the levels of reporter gene expression are approximately 1,000-fold higher than those observed in the absence of viral sequences (41). Removal of certain clones from this genomic library results in greater than 20-fold decreases in transactivation levels. By replacing individual or overlapping sets of clones from the genomic library with subclones capable of supplying the transactivating activity, a set of 18 plasmids which support transient late gene expression in the IPBL-SF-21 (SF-21) cell line derived from the fall armyworm *Spodoptera frugiperda* was identified (23, 27, 33, 38–42, 50, 51). This set of 18 plasmids, each containing an identified late expression factor gene (*lef*), is designated the *lef* library. Very late gene expression, which is required for occlusion body formation, additionally requires the function of the very late expression factor 1 gene (*vlf-1*) product, a polypeptide with sequence motifs characteristic of a family of integrases/resolvases (30, 50).

A subset of *lef* genes, *ie-1*, *ie-2*, *lef-1*, *lef-2*, *lef-3*, *p143*, *dnapol*, *p35*, and *lef-7*, are required for optimal *hr*-dependent plasmid DNA replication in SF-21 cells and are therefore known as replication *lef* genes (20, 28). The need for replication *lef* genes demonstrates the dependence of late gene expression on DNA replication or reporter plasmid stability in the transient expression system (20, 28). Expression of late and very late baculovirus genes also depends on viral DNA replication during infection (25, 46, 49). IE-1 transactivates early gene expression and may be involved in transactivating *lef* gene expression (15, 16, 34). However, it also recognizes and binds imperfect palindromes within *hr* regions and may therefore be directly involved in DNA replication (8, 14, 47). IE-2 has two known functions: transactivating early gene expression (4, 5) and blocking cell cycle progression (44). IE-2 is not required for transient late gene expression in TN-368 cells (26) derived from *Trichoplusia ni*, and its role in the SF-21 assays is not clear. LEF-1 contains three sequence motifs which are conserved in DNA primases, suggesting that LEF-1 may be a baculovirus primase (2, 11). LEF-2 is known to be an essential gene for Ac*M*NPV replication, and some mutant alleles display a delay in late gene expression and a defect in very late gene expression (31). LEF-3 is a single-stranded DNA binding protein (17). *p143* and *dnapol* encode polypeptides with sequence similarities to DNA helicases and DNA polymerases, respectively. p35 is a general caspase inhibitor which is required to block virus-induced apoptosis in SF-21 cells (3, 9), and its role in this assay may be to stabilize the reporter plasmid from nucleolytic degradation (50). LEF-7, like IE-2 and p35, has little or no influence on plasmid DNA replication or stability in TN-368 cells, suggesting cell line-specific or host-specific factors are required for *hr*-dependent DNA replication or stability (26). In the cases of *p35* and *lef-7*, such host or cell line specificity has been confirmed by analysis of mutant virus phenotypes (7, 9). An additional replicative *lef*, *hcf-1*, is required for optimal *hr*-dependent DNA replication and transient late gene expression in TN-368 cells (26), and virus mutants with deletions in *hcf-1* show delayed DNA replication and defective late gene expression in TN-368 cells (29).

The remaining nine *lef* genes, termed transcription-specific *lef* genes, affect the steady-state levels of reporter gene transcripts but not plasmid DNA and are thus likely to be involved in transcription or RNA processing and stability (28). Two of these genes (*lef-8* and *lef-9*) are predicted to be components of a viral RNA polymerase, based on amino acid sequence motifs that are conserved in prokaryotic and eukaryotic RNA polymerases (27, 42), and *lef-6* may have a sequence motif related to one found in vaccinia virus RNA polymerase (39). The roles of p47 and LEF-4 in late gene transcription were identified by

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TABLE 1. Oligonucleotides and plasmid templates used to construct the HSEpiHis *lef* genes

Primer	Sequence[a]	GenBank coordinates[b]	Template	Reference
NIE1	5′-GAAGATCTACGCAAATTAATTTTAACGC-3′	127201–127220	pPS-IE1/HC	41
CIE1	5′-TTTTCCCCCCGGGTCGCCAACTCCCATTGTTAT-3′	129048–129067	pPS-IE1/HC	41
N2IE2	5′-CGCGGATCCAGTCGCCAAATCAACGCCGCC-3′	132060–132080	pBSP-PstNA	41
CIE2	5′-AAGGAAAAAAGCGGCCGCTGGATGTACCGCTAACCAAA-3′	130750–130731	pBSP-PstNA	41
NLF2noIM	5′-GAAGATCTGCGAATGCATCGTATAACGT-3′	3092–3111	pBE42-630	41
CLEF2	5′-TTTTCCCCCCGGGATTTATAATTGTTTTATTAT-3′	3722–3741	pBE42-630	41
NLEF1	5′-GAAGATCTTTAGTGTGCAATTATACGCA-3′	11291–11310	pBS BCNE	38
CLEF1	5′-TTTTCCCCCCGGGTTGCATTTGAATGAGTCCCA-3′	10463–10483	pBS BCNE	38
NLEF6	5′-GAAGATCTAACATGGTGTTCAACGTGTACTAC-3′	23465–23485	pAcIAP-PstI/NsiI	39
CLEF6	5′-TACTCCCGGGGTTTTATTGTTTTTCTAATACATTC-3′	23965–23989	pAcIAP-PstI/NsiI	39
N39K	5′-GAAGATCTAACATGGTAAACGTGCCGGAG-3′	30052–30072	pNspAfl	51
C39Klong	5′-TTTCCCCCCGGGTTAATCTGACATATTTGTAT-3′	29242–29261	pNspAfl	51
NLEF11	5′-GAAGATCTAACATGCCCCCCAAAAATTGCAC-3′	30382–30401	pHindIIIR	51
CLEF11	5′-ATATCCCGGGTGTTGCTCCGGCACGTTTAC-3′	30047–30066	pHindIIIR	51
N2P47	5′-GAAGATCTTTTGTCACCCGGTTGGAGCACA-3′	33358–33379	p47*	51
CP47	5′-ATATCCCGGGTTTGTCCATGATTGGCTCAG-3′	32161–32180	p47*	51
NLEF12	5′-GAAGATCTACTATGGAAAATAACGCGGAATTCA-3′	33384–33408	p47*	51
CLEF12	5′-TTTTCCCCCCGGGTATTGCGCGCTCGTAATCTA-3′	33954–33973	p47*	51
N2LEF8	5′-GAAGATCTACGGACGTGGTTCAAGATTT-3′	43131–43150	pBS-RI-M	42
CLEF8LONG	5′-TTTCCCCCCGGGCAGTGATTCTAATTGCAGCTGC-3′	40420–40441	pBS-RI-M	42
N2LEF10	5′-GAAGATCTACGAACGTATGGTTCGCGACGG-3′	45131–45152	pPstHIME0.5	27
CLEF101g	5′-TTTCCCCCCGGGAATAATTGTTACGTGGACGCG-3′	45352–45372	pPstHIME0.5	27
N2LEF9	5′-GAAGATCTTTTTCTTTTTTGGATAAAACTCC-3′	49265–49286	pPstHISB2.35	27
CLEF9LONG	5′-TTTCCCCCCGGGTTATCATTCAATGAACATGTCG-3′	50716–50737	pPstHISB2.35	27
NDNAp	5′-GAAGATCTAAAATATATCCTTACAATGA-3′	55261–55280	pDNAp	27
CDNAp	5′-AAGGAAAAAAGCGGCCGCAACCGCTGTCGTAATCTTGG-3′	52227–52246	pDNAp	27
N2LEF3	5′-CGCGGATCCGCGACCAAAAGATCTTTGTC-3′	58856–58875	pSDEM2	23
C2LEF3	5′-TTTTCCCCCCGGGTTGCAGATCAGGCTGTCAAA-3′	57534–57553	pSDEM2	23
5′ hcflbgl	5′-GAAGATCTGATTCGCTAGCCAATTTGTGCTTG-3′	60113–60136	pXABgE3.6	26
3′ hcflpspa	5′-TTTTCCCCCCGGGTCTAGCGACATGTTTGCC-3′	61075–61092	pXABgE3.6	26
NVLF1	5′-GAAGATCTAACGGTTTTAATGTTCGCAA-3′	64930–64949	pXA-7	30
CVLF1	5′-TTTCCCCCCGGGCCGGGCTGACGATAATAAAC-3′	63677–63696	pXA-7	30
NLEF4	5′-GAAGATCTAACATGGACTACGGCGATTTTG-3′	76596–76614	p86D5B	40
CLEF4	5′-TATCCCGGGGCTTTAATTTGGCACGATTC-3′	77974–77992	p86D5B	40
p143N	5′-GAAGATCTATTGACAACATTTTACAATT-3′	84337–84356	pBR322-RI-D/SmaIΔORF6	40
p143C	5′-TTTTCCCCCCGGGATTGTGTGTTTGATCGACCC-3′	80460–80479	pBR322-RI-D/SmaIΔORF6	40
NLEF5	5′-GAAGATCTAACATGTCGTTTGATGATG-3′	85918–85933	pH3H/SH/ORF6	40
CLEF5	5′-TATCCCGGGCTATTAACAACCAGACATTCC-3′	86698–86718	pH3H/SH/ORF6	40
NLEF7	5′-GAAGATCTTCGAGCGTTACAAAGCGCCC-3′	105211–105230	pBSXBglII	33
CLEF7	5′-TTTTCCCCCCGGGCGCCACCGTTCTCTAACATT-3′	104487–104506	pBSXBglII	33

[a] Restriction sites used for cloning into pHSEpiHisVI^{+} are underlined.
[b] For accession no. L22858 (1).

analysis of conditional lethal mutants of Ac*M*NPV (6, 37). These mutants synthesize viral DNA at the nonpermissive temperature but are defective in late and very late gene expression. The remaining transcription-specific genes, *lef-5*, *39k*, *lef-10*, and *lef-11*, appear to have no significant homology to genes in existing sequence databases.

Plasmids of the *lef* library supply the 18 *lef* genes, but because of additional flanking sequences included during subcloning, the *lef* library collectively contains approximately one-third of the Ac*M*NPV genome. To define the role of each *lef* gene more thoroughly, it was of interest to construct a library of just the open reading frames (ORFs) of the *lef* genes. Furthermore, the genes comprising the *lef* library are all under the control of their original promoters, and at least some of them are known to be transactivated by IE-1 and/or IE-2 (5, 15, 24, 33–35). Therefore, we constructed a set of plasmids, each containing only a single *lef* ORF, fused to epitope and His_6 tags and placed under constitutive promoter control. This collection of plasmids is designated the HSEpiHis *lef* library. In the process of constructing the HSEpiHis *lef* library, we discovered a previously unidentified *lef*, *lef-12*, on the plasmid subclone p47. The p47 plasmid is subsequently referred to as p47* since it contains two *lef* genes (*p47* and *lef-12*) which are required for late and very late gene expression. We also found that all 19 *lef* genes are necessary for transient late gene expression in SF-21 cells and that collectively these genes are sufficient to support late gene expression at levels that are 30% or higher than that observed in the presence of the entire genome.

MATERIALS AND METHODS

Cells. *S. frugiperda* SF-21 (53) and *T. ni* TN-368 (19) cells were grown at 27°C in TC-100 medium (Life Technologies, Inc., Gaithersburg, Md.) containing 10% fetal bovine serum and 0.26% tryptose broth (36).

Plasmid constructs. The previously described reporter plasmids pETCAThr5 (41), pCAPCAT (49), and phcwt (45) contain the early ETL, late *vp39*, and very late *polh* promoters, respectively, controlling the reporter gene, which encodes chloramphenicol acetyltransferase (*cat*). The construction of pHSEpip35VI^{+} has been described elsewhere (48).

The *lef* library and overlapping Ac*M*NPV clone library used in these experiments (Table 1) have been described elsewhere (41).

Plasmid pHSEpiHisVI^{+} was constructed by digesting pHSEpiOpIAPVI^{+} (18) with *Xma*I and *Not*I to remove the OpIAP ORF and inserting overlapping oligonucleotides with *Xma*I and *Not*I cohesive ends encoding a His_6 tag and *Bgl*II, *Psp*AI, and *Not*I sites. A schematic diagram of pHSEpiHisVI^{+} with a generic *lef* inserted in the *Bgl*II and *Psp*AI sites is shown in Fig. 1. In the name pHSEpiHisVI^{+}, "HS" refers to the *Drosophila melanogaster* heat shock protein 70 (hsp70) promoter (52), "Epi" refers to the HA.11 epitope (12), and "His" refers to the six-histidine tag fused to the N terminus of the *lef* gene cloned into the vector; "VI^{+}" indicates that the vector contains the polyhedrin gene and sequences flanking this gene which allow selectable homologous recombination into the baculovirus genome for expression in future studies.

To construct the HSEpiHis *lef* library, the ORF for each *lef* was amplified with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.), using the primers and plasmid templates of the *lef* library listed in Table 1. The N2LEF9 primer corre-

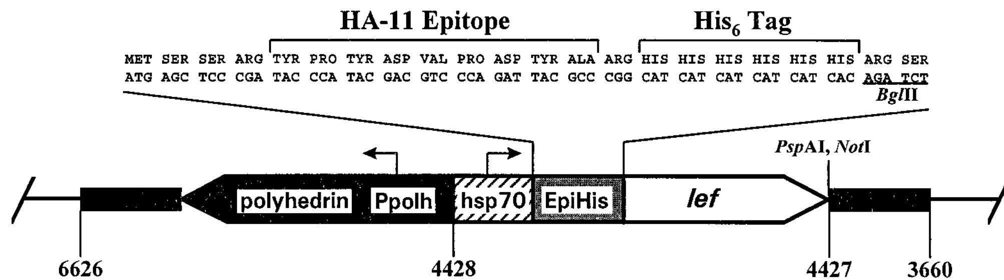

FIG. 1. Schematic diagram of pHSEpiHisVI$^+$. Arrows indicate the direction of transcription of the polyhedrin gene and *D. melanogaster* hsp70 gene promoters (Ppolh and hsp70, respectively). "EpiHis" refers to the HA.11 epitope and His_6 tag which are fused to the N terminus of each *lef*. Only the *Bgl*II, *Psp*AI, and *Not*I sites of the multiple cloning site are shown. The orientation of a generic *lef* inserted between the *Bgl*II and *Psp*AI sites is indicated by an open arrow. Black portions indicate the polyhedrin gene and flanking viral sequences. The limits of the viral sequences in this vector are indicated by the numbers at the bottom (1) (GenBank accession no. L22858). The pUC8 sequences are represented by the single black line.

sponded to the second ATG of the *lef-9* ORF. PCR products were digested with the appropriate restriction enzymes (sites underlined in Table 1) and gel purified prior to their insertion into pHSEpiHisVI$^+$. The 5′ end of each *lef* PCR product was ligated to the *Bgl*II site of the pHSEpiHisVI$^+$ multiple cloning site (Fig. 1).

Construction of *lef-12* frameshift mutants. The Ac*M*NPV plasmid subclone p47* was digested with *Eco*RI; the ends were filled in with T4 polymerase and religated to produce plasmid p47*/*Eco*RI fs. The mutation in this plasmid was confirmed by sequencing, and the mutant gene is predicted to produce a 73-amino-acid peptide containing the first 9 amino acids of *lef-12*. To construct p47*/*Apa*I fs, p47* was digested with *Apa*I, and the ends were removed by using T4 DNA polymerase and religated. This plasmid, which was sequenced and found to contain a 5-bp deletion in the *lef-12* ORF, is predicted to produce a 106-amino-acid peptide that contains the first 95 amino acids of *lef-12*. The same mutations were made in the plasmid containing the hsp70-promoted *lef-12* ORF (pHSEpiHis *lef-12*) and confirmed by sequencing; the resultant plasmids were called pHSEpiHis *lef-12*/*Eco*RI fs and pHSEpiHis *lef-12*/*Apa*I fs.

Transfections and transient expression assays. SF-21 or TN-368 cells were transfected by using Lipofectin reagent (Life Technologies). Cells were transfected with 2.0 μg of reporter plasmid and either 0.5 μg of each of the clones of the Ac*M*NPV genomic library or 1.0 μg of each plasmid of the *lef* library or HSEpiHis *lef* library.

Cells were collected at 24, 48, and 72 h after transfection for samples containing pETCAThr5, pCAPCAT, and phcwt, respectively, and lysates were assayed for CAT activity (13), using 1/10 of the lysate or dilutions thereof for quantitation purposes. Quantitations of CAT assays were done directly on the thin-layer chromatography plates with a PhosphorImager 4000 (Molecular Dynamics, Sunnyvale, Calif.).

DNA replication assays. The method has been described previously (28). Briefly, 1.8×10^6 SF-21 cells were cotransfected with pCAPCAT and clones of the Ac*M*NPV genomic library or the HSEpiHis *lef* library. DNA from each sample was digested with *Bgl*II and *Dpn*I, electrophoresed through a 0.7% agarose gel, and transferred to Zeta-probe nylon membranes (Bio-Rad, Richmond, Calif.). Membrane-bound DNA was hybridized to a [^{32}P]dCTP nick-translated *Bgl*II-*Kpn*I fragment of pCAPCAT containing the *cat* ORF. The relative levels of DNA replication were determined by using a PhosphorImager 4000.

Immunoblotting. SF-21 cells (5.4×10^5) were transfected with 2 μg of each HSEpiHis *lef* as described above. At 14 h after transfection, the cells were heat shocked for 30 min at 42°C. At 2 h after heat shock treatment, cells were lysed in sodium dodecyl sulfate (SDS) sample buffer, and equal volumes representing the same cell number were resolved on an SDS–10 to 18% gradient polyacrylamide gel. The resolved proteins were then blotted onto polyvinylidene fluoride membranes (Millipore, Bedford, Mass.). The membranes were blocked and then incubated with anti-HA.11 mouse immunoglobulin G (BAbCO, Richmond, Calif.) followed by anti-mouse immunoglobulin G conjugated to horseradish peroxidase. Immunoreactive proteins were visualized with the enhanced chemiluminescence Western blotting system (Amersham Life Science Inc., Arlington Heights, Ill.).

RESULTS

Construction of the HSEpiHis *lef* library. We constructed a *lef* library (HSEpiHis *lef* library) in which all of the individual *lef* ORFs were placed under *D. melanogaster* hsp70 promoter control within plasmid pHSEpiHisVI$^+$ (Fig. 1). The hsp70 promoter was chosen for expression of *lef* genes since earlier studies have shown that the promoter is constitutively expressed in the absence of heat shock in SF-21 and TN-368 cells (32) but can be strongly induced with heat shock treatment (10).

ORF41 (*lef-12*) is involved in late gene expression in SF-21 cells. CAT gene expression from the late promoter of reporter plasmid pCAPCAT is activated over 100-fold by cotransfection with the set of genomic clones representing the entire Ac*M*NPV genome (Fig. 2A; compare lanes 1 and 2). Similarly, addition of the 18 clones constituting the *lef* library stimulates expression over 100-fold (lane 3). The level of expression from the *lef* library is three- to fourfold lower than that from the genomic library in this experiment. In other experiments using other preparations of plasmids, the two libraries can be virtually equivalent (28), although the general trend is for expression from the *lef* library to be slightly lower than that from the genomic library. Since we define *lef* genes as those genes which provide at least a 10-fold stimulation to late reporter gene expression, the *lef* library appears to contain all the necessary *lef* genes for late gene expression but may lack genes which can stimulate expression mildly. Deletion of any one of the *lef* clones from the library decreases expression by 10-fold or more (e.g., *lef-1* [lane 4]) (28).

Each plasmid of the HSEpiHis *lef* library was tested for its ability to substitute in transient expression assays for its counterpart in the *lef* library. A representative example of how each HSEpiHis *lef* was tested is shown for HSEpiHis *lef-1*, which restored the relative CAT activity to levels observed with the *lef* library containing *lef-1* (Fig. 2A, lanes 3 through 5). Unlike the other 17 HSEpiHis *lef* genes which could substitute for their counterpart in the *lef* library in the transient assay, HSEpiHis *p47* did not restore CAT expression to the level observed with the *lef* library containing p47* (lanes 6 and 7). Therefore, a set of frameshift mutants of p47* (Fig. 2B) was used to investigate the possibility that p47* contained an unidentified *lef* (Fig. 2A, lanes 8 through 15). In the absence of the p47* plasmid but in the presence of HSEpiHisp47, the addition of p47*/*Xba*I fs, which is defective in *p47* function (51), restored CAT activity to levels similar to those observed with the *lef* library (lane 13), suggesting that p47* contains a second element required for transient late gene expression. The p47* plasmid, formerly known as p47, contains 911 bp upstream and 307 bp downstream of the *p47* ORF (Fig. 2B). One other ORF, ORF41, is present on p47*; this ORF has the potential to encode a polypeptide of at least 50 amino acids and could supply *lef* function in the assay. In the absence of p47*, frameshift mutants of ORF41, p47*/*Eco*RI, and p47*/*Apa*I did not restore expression of the reporter plasmid (Fig. 2A, lanes 9 and 10) indicating that ORF41 had transactivating activity and/or that the *Eco*RI and *Apa*I mutations interfered with the expression of *p47*. The possibility that the frameshift mutations in ORF41 were interfering with *p47* expression was excluded since in the absence of p47*, both p47*/*Eco*RI and p47*/*Apa*I

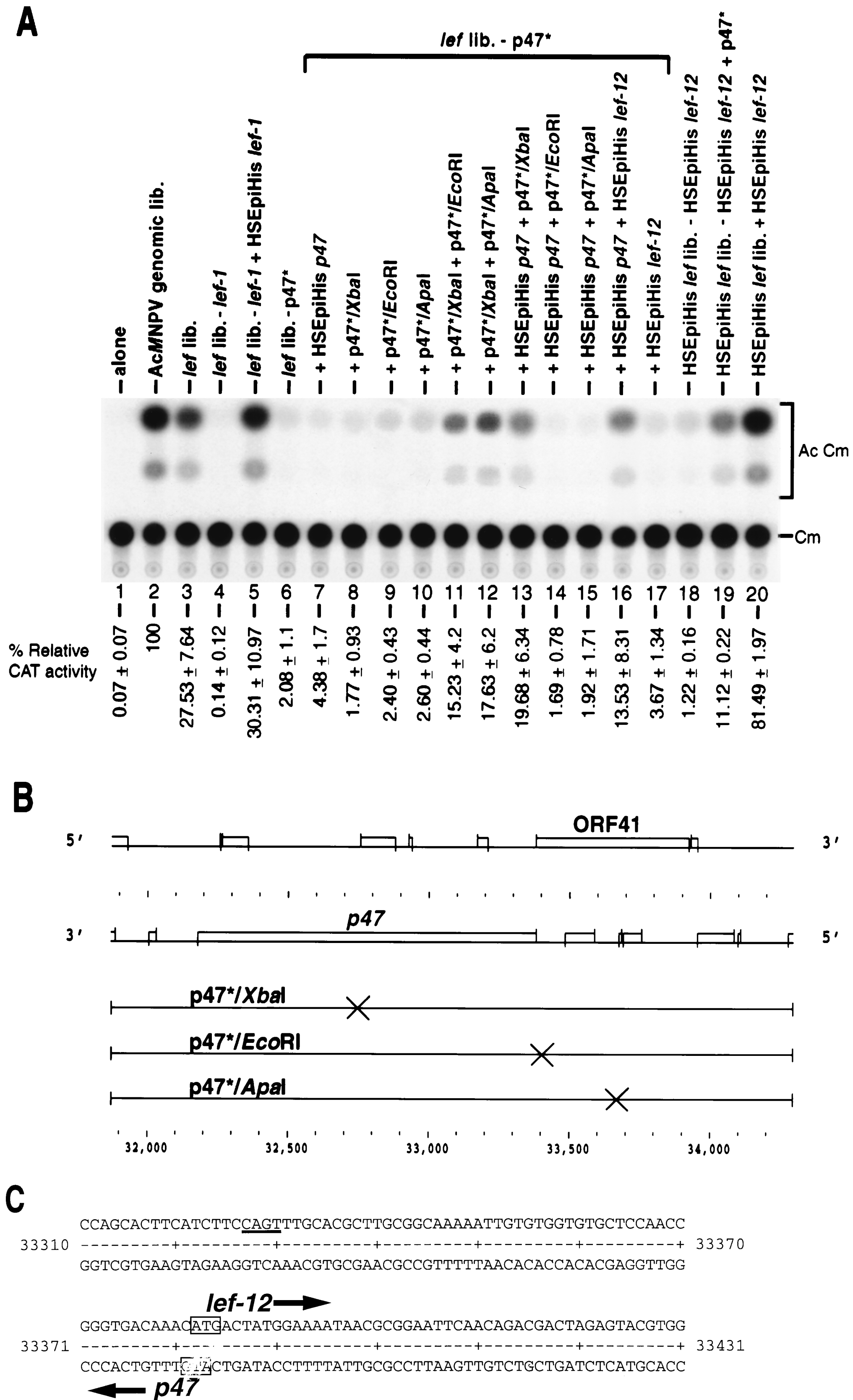

FIG. 2. Involvement of ORF 41 (*lef-12*) in late gene expression. (A) Transient expression assays showing the levels of CAT activity from SF-21 cells transfected with the late reporter plasmid pCAPCAT alone (lane 1) or cotransfected with pCAPCAT and the complete Ac*M*NPV genomic library (lane 2), the *lef* library (lane 3), the *lef* library with *lef-1* omitted (lanes 4 and 5), the *lef* library with p47* omitted (lanes 6 through 17), or the HSEpiHis *lef* library (lanes 18 through 20). Additional plasmid clones or clones missing from cotransfections are shown above each lane. Lanes 4 and 6 contained the empty vector plasmid pHSEpiHisVI^{+} to maintain DNA

were able to restore CAT activity when used in combination with p47*/*Xba*I (which is defective in *p47*) and thus were able to supply *p47* activity (lanes 11 and 12). In addition, ORF41 was cloned into pHSEpiHisVI$^+$ and tested in the *lef* assay in combination with HSEpiHis *p47* (lane 16) or without the *p47* ORF (lane 17). CAT activity was partially restored when both the *p47* and ORF41 coding sequences were expressed constitutively in the context of the *lef* library (lane 16). In the context of the HSEpiHis lef library, the coding sequence of ORF41, subsequently known as *lef-12*, transactivated expression from the *vp39* promoter significantly and gave levels of CAT activity 80% of those seen for the Ac*M*NPV genomic library (lanes 18 through 20). It is possible that *lef-12* down regulates one or more *lef* promoters while transactivating the *vp39* promoter, and thus its effect is most easily observed in the context of the HSEpiHis *lef* library.

Examination of the sequence of p47* revealed that the translational initiation codons for the *p47* ORF and the *lef-12* ORF divergently overlap by two nucleotides, based on the predicted ORFs described by Ayres et al. (1) (Fig. 2C).

Immunoblot detection of each HSEpiHis *lef*. To test the expression of each HSEpiHis *lef*, plasmids were transfected individually into SF-21 cells; the cells were subsequently heat shocked, and the cell lysates were subjected to immunoblot analysis (Fig. 3). Predicted molecular masses of the HSEpiHis proteins ranged from 11.1 kDa for HSEpiHis *lef-10* (Fig. 3, lane 11) to 146 kDa for HSEpiHis p143 (lane 18). Although equal portions of cell lysates representing equal numbers of cells were loaded on the gel for each HSEpiHis *lef* gene, expression levels varied widely. HSEpiHis *lef-10* (Fig. 3, lane 11) reproducibly showed the lowest level of expression among the HSEpiHis *lef* genes. In contrast, HSEpiHis *lef-3* (lane 14) and HSEpiHis *lef-12* (lane 9) reproducibly showed the highest levels of expression and were easily detectable.

***lef-12* is involved in late gene expression in the context of the overlapping genomic library in SF-21 cells.** Since *p47* was originally identified as the only late expression factor within p47* responsible for transient late gene expression in the context of the Ac*M*NPV genomic library, we reexamined the roles of both *p47* and *lef-12* in this context. The genes for *lef-12* and *p47* are located on two adjacent overlapping genomic clones, HL5 (starting at bp 22060 and ending at bp 38451) and ETL7 (starting at bp 29032 and ending at bp 45037) (1). In addition to *p47* and *lef-12*, HL5 and ETL7 contain copies of the genes for late expression factors *lef-6*, *39k*, *lef-11*, and *lef-8*. However, unlike *p47* and *lef-12*, one or more copies of each of these *lef* genes are present on other library clones. To investigate the influence of *lef-12* in transient late gene expression assays with the Ac*M*NPV genomic library, we removed HL5 and ETL7 and replaced them with plasmids containing intact or frameshift versions of *lef-12* and *p47* (Fig. 4A). Omission of HL5 and ETL7 from the Ac*M*NPV library reduced CAT gene expression to background levels (Fig. 4A; compare lanes 2 and 3). The addition of p47*, which contains both *p47* and *lef-12*, increased CAT expression levels to 140% of that seen using the Ac*M*NPV genomic library (lane 4); thus, sufficient quantities of *lef-6*, *39k*, *lef-11* and *lef-8* are expressed from genes located on other genomic clones, and p47* was able to provide the remaining *lef* genes found in the Ac*M*NPV genomic library. Substitution of p47* with a plasmid containing a frameshift mutant of *p47* (p47*/*Xba*I) reduced CAT gene expression to background levels (lane 5) and supports the previous finding that *p47* is required for optimal late gene expression (51). Substituting p47* with clones containing intact *p47* and frameshifts of *lef-12* (p47*/*Eco*RI and p47*/*Apa*I) reduced CAT gene expression to approximately 28% of that seen with wild-type *lef-12* (compare lanes 6 and 7 with lane 4) and to about 40% of that seen with the complete genomic library (compare lanes 6 and 7 with lane 2). This finding indicated that *lef-12* has a role in late gene expression in transient assays using the genomic library but does not appear to be required in this context.

In assays using the Ac*M*NPV genomic library, substitution of clones HL5 and ETL7 with two plasmids, p47*/*Xba*I, the plasmid containing a mutant *p47* but an intact *lef-12*, and either p47*/*Eco*RI or p47*/*Apa*I, both of which contain a mutated *lef-12* and an intact *p47*, did not give high levels of CAT expression (Fig. 4A; compare lanes 8 and 9 with lanes 6 and 7). The reason for the lack of complementation between these plasmids in this assay is not clear.

As demonstrated in Fig. 2A, *lef-12* was required for optimal late gene expression in transient assays using a set of 19 plasmids in the context of the HSEpiHis *lef* library. Removal of pHSEpiHis *lef-12* or substitution with a pHSEpiHisVI$^+$-based plasmid containing a frameshift of *lef-12* or a *lacZ* gene reduced CAT gene expression to background levels (Fig. 4B; compare lanes 3 to 6 with lane 2), confirming that *lef-12* is a late expression factor in this context.

LEF-12 has a stimulatory role on late gene expression in TN-368 cells in the context of the HSEpiHis *lef* library. In transient late expression assays of TN-368 cells, when clones HL5 and ETL7 were removed from the genomic library and *p47* and *lef-12* were provided by the addition of p47*, CAT expression levels were only 15 to 20% of that seen for the complete Ac*M*NPV genomic library (data not shown). To eliminate the possibility that the reduced copy numbers of the other *lef* genes present on HL5 and ETL7 limited CAT expression, the assays were supplemented with plasmids containing *lef-6*, *39k*, *lef-11*, and *lef-8*. The level of CAT gene expression increased marginally to about 25 to 30% of that seen with the complete genomic library (Fig. 4A, lane 4). The reason why p47* cannot fully substitute for HL5 and ETL7 in TN-368 cells is not known. Substituting a frameshift mutant *p47* in this system resulted in background levels of CAT expression (Fig. 4C; compare lane 5 with lane 1), confirming a role for *p47* in late gene expression in transient assays in TN-368 cells. Lack of a functioning *lef-12* gene caused only a 40 to 50% reduction in the levels of CAT gene expression (compare lanes 6 and 7 with lane 4) and suggested that *lef-12* may play only a stimulatory role in TN-368 cells. As in SF-21 cells, addition of two plasmids each containing a frameshift in *p47* or *lef-12* did not further augment expression (lanes 8 and 9).

In TN-368 cells, removal of pHSEpiHis *lef-12* from the HSEpiHis *lef* library or replacement with a construct containing a pHSEpiHisVI$^+$-based frameshifted *lef-12* gene or *lacZ* gene reduced CAT gene expression by a factor of 3 (Fig. 4D; compare lanes 3 to 5 with lane 2). This contrasts with the 10-fold

concentrations equivalent to those in lanes 5 and 7. The acetylated chloramphenicol products (Ac Cm) and unacetylated substrate (Cm) are indicated on the right. Relative CAT activities are shown below each lane. (B) ORFs and frameshift constructs of p47*. The ORF diagram was generated by the FRAMES program of the Wisconsin Package, version 8.1 (Genetics Computer Group, Inc., Madison, Wis.). Frameshift mutations introduced into p47* (×) are indicated on the *Xba*I, *Eco*RI, and *Apa*I sites. Plasmid p47*/*Xba*I has been previously described (51). Numbers at the bottom refer to sequence coordinates for GenBank accession no. L22858 (1). (C) Nucleotide sequence of the *p47* and *lef-12* overlapping N-terminal regions. Initiating methionine codons are boxed. Large arrows show the direction of the *lef-12* and *p47* ORFs. A possible initiation site for *lef-12* transcription is underlined. Numbers at the sides refer to coordinates for GenBank accession no. L22858 (1).

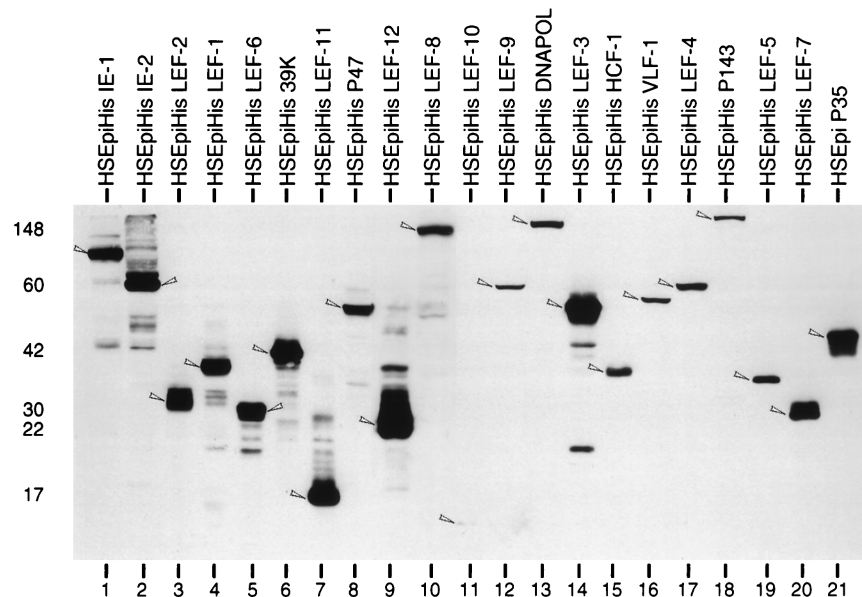

FIG. 3. Immunoblot of HSEpiHis *lef* genes expressed in transfected cells. SF-21 cells were transfected with 2 μg of each HSEpiHis *lef*, heat shocked for 30 min at 18 h posttransfection, and harvested 2 h after heat shock. Equal amounts of total cell lysates were analyzed on an SDS–10 to 18% gradient polyacrylamide gel followed by immunoblot analysis using an anti-hemagglutinin monoclonal antibody. Arrowheads indicate the bands corresponding to the predicted molecular weight of each HSE piHis LEF. Positions of molecular weight standards (in thousands) are shown on the left.

reduction seen in SF-21 cells and suggests that *lef-12* has a stimulatory rather than an essential role in late transient gene expression in TN-368 cells.

***lef-12* is involved in late and very late but not early gene expression.** Since HSEpiHis *lef-12* was important for expression from the late reporter plasmid, pCAPCAT, in combination with the HSEpiHis *lef* library in SF-21 cells, it was of interest to determine whether HSEpiHis *lef-12* had an effect on early and very late gene expression. To test this, HSEpiHis *lef-12* was cotransfected with the HSEpiHis *lef* library containing all of the *lef* genes except HSEpiHis *lef-12* along with reporter plasmids containing the CAT gene under early (pETCAThr5) or very late (phcwt) promoter control (Fig. 5). Expression of pETCAThr5 was unaffected by replacement of HSEpiHis *lef-12* by its frameshifted version (Fig. 6; compare lanes 1 and 2), indicating that *lef-12* does not stimulate expression from this early promoter. In contrast, substitution of the frameshifted HSEpiHis *lef-12* resulted in dramatic decreases in CAT expression from both pCAPCAT and phcwt (compare lanes 3 and 4 and lanes 5 and 6, respectively), indicating the involvement of a functional HSEpiHis *lef-12* gene product in both late and very late gene expression.

Effect of omitting each EpiHis *lef* library clone on expression from a late promoter in SF-21 cells. To determine the specific contribution of each HSEpiHis *lef* to late gene expression from the reporter plasmid pCAPCAT, each *lef* was removed individually from cotransfections which included all other members of the HSEpiHis *lef* library and replaced with the control vector plasmid pHSEpiHisVI$^+$ (Fig. 6). Removal of HSEpiHis *ie-2* resulted in only a 3.7-fold decrease in relative CAT activity (Fig. 6, lane 5 versus lane 3), while individual removal of the other HSEpiHis *lef* genes, such as HSEpiHis *lef-12* (lane 12), resulted in a 35-fold or greater decrease in relative CAT activity (lanes 4 and 6 through 22).

***lef-12* does not play a role in DNA replication.** In previous experiments which identified late expression factors with roles in DNA replication, it was established that p47* was not required in *hr*-dependent plasmid replication assays. Since *lef-12* was also provided by p47*, it is likely that like *p47*, *lef-12* is not required for DNA replication. However, it was of interest to investigate whether *lef-12* had any effect on DNA replication in the context of the pHSEpiHis *lef* replication library. In transient DNA replication assays, the pHSEpiHis *lef* replication library gave levels of replication approximately 80% of those seen with the Ac*M*NPV genomic library (Fig. 7, lanes 2 and 3). When pHSEpiHis *lef-12* was added to the pHSEpiHis replication *lef* library, there was no significant difference in the levels of plasmid DNA replication (lane 4). To determine the specific contribution of each HSEpiHis *lef* on transient DNA replication, we removed each HSEpiHis *lef* individually from the assay system. Removal of HSEpiHis *ie-1*, *lef-2*, *lef-1*, *lef-3*, *p143*, and *p35* (lanes 5 and 7 through 10) strongly reduced replication levels, indicating a role in viral DNA replication, whereas removal of pHSEpiHis *ie-2*, pHSEpiHis *dnapol*, and pHSEpiHis *lef-7* (lanes 6, 11, and 13) decreased transient replication levels partially, supporting the view that these *lef* genes are stimulatory for DNA replication under these transient assay conditions (28).

DISCUSSION

We have constructed a set of 18 plasmids in which each previously identified *lef* ORF is expressed as an epitope-tagged fusion protein from the *D. melanogaster* hsp70 promoter. Failure of this library to support late gene expression in transient expression assays in SF-21 cells led to the identification of a 19th *lef*, *lef-12* (ORF41), located adjacent to *p47*. The set of 19 plasmids, designated the HSEpiHis *lef* library, supports gene expression from a late viral promoter in transient assays.

lef-12 potentially encodes a 181-amino-acid polypeptide with a predicted molecular mass of 21,058 Da with no significant similarities to other genes in the protein or nucleic acid databases. *lef-12* is predicted to be expressed as an early gene, since a CAGT motif is found at an appropriate distance (52 bp) up-

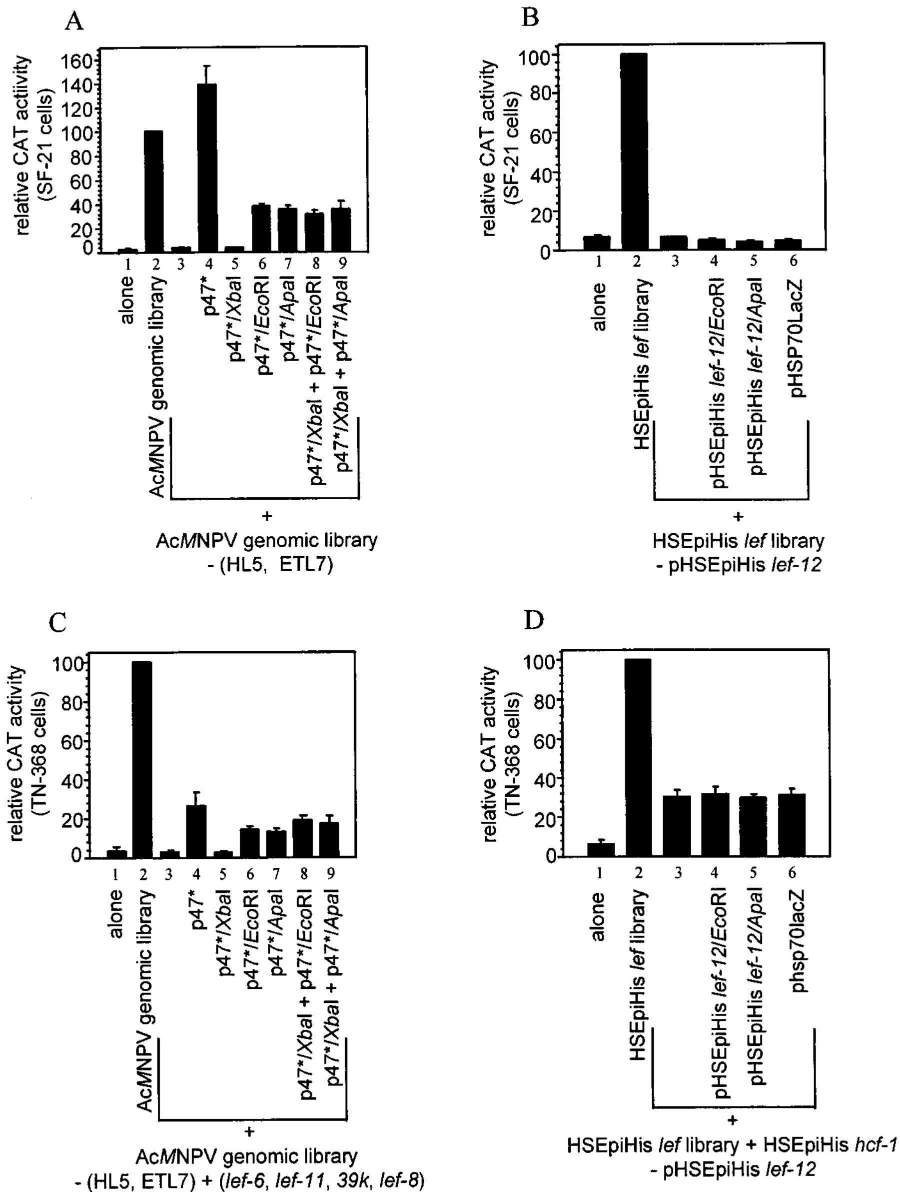

FIG. 4. Requirement for *lef-12* in transient late gene expression assays. SF-21 cells (A and B) or TN-368 cells (C and D) were transfected with the Ac*M*NPV genomic library (A and C) or the HSEpiHis *lef* library (B and D). Library clones supplied or omitted from the complete libraries are indicated below each graph. In panel C, plasmid clones pAcIAP-PstI/NsiI and pBS-RI-M were added to supply *lef-6* and *lef-8*, respectively, and plasmid pFspAfl (51) was added to supply both *39k* and *lef-11*. Plasmids supplying wild-type *p47* or *lef-12* or corresponding frameshift mutations are shown below the lanes. CAT activities are reported relative to those of the full libraries; data represent the mean of at least three independent experiments, and bars represent the standard error.

stream from the *lef-12* initiation codon, and the nearest TAAG sequence, a characteristic promoter element of late and very late genes, is 711 bp upstream from the *lef-12* initiation codon.

In the context of the HSEpiHis *lef* library in SF-21 cells, *lef-12* is necessary for expression of a reporter gene under control of the late capsid or very late polyhedrin promoters but does not affect the early ETL promoter. Although *lef-12* activates late gene expression at least 10-fold in the context of the HSE piHis *lef* library in SF-21 cells, it exerts only a 3-fold effect in TN-368 cells in this context. Whether this gene is essential to Ac*M*NPV infection remains to be determined.

The difference in the relative level of stimulation by *lef-12* observed in the two contexts, the Ac*M*NPV genomic library or the HSEpiHis *lef* library, suggests that another Ac*M*NPV gene may be functionally redundant with *lef-12*, that *lef-12* may negatively affect transcription from another *lef* promoter or that the relative levels of expression of the *lef* genes may influence gene expression in these transient assays. As shown in Fig. 3, the relative levels of expression of members of the HSEpiHis *lef* library varied widely and probably do not reflect the levels of *lef* expression found in an Ac*M*NPV infection or in transient assays involving the Ac*M*NPV genomic library.

Clues to why *lef-12* was not identified in earlier experiments (51) may be obtained from the failure of a pair of plasmid subclones, one containing an intact *p47* and the other containing an intact *lef-12*, to substitute for plasmid p47* in the Ac*M*NPV genomic library. The presence of both plasmids should have provided intact copies of both *p47* and *lef-12* and thus restored activity, but they did not. The reason these plas-

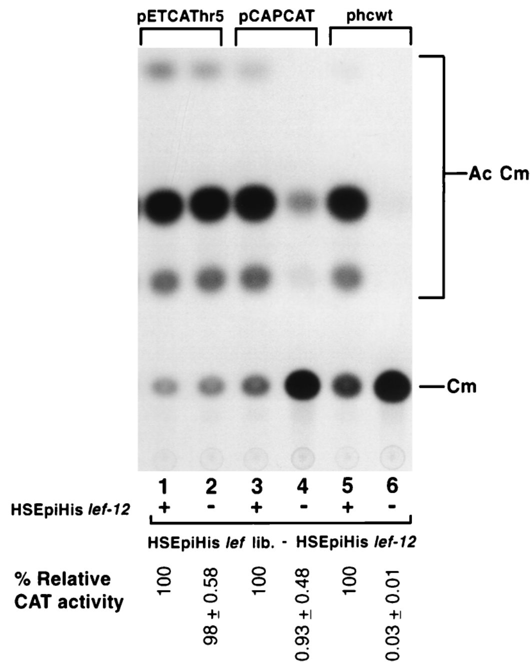

FIG. 5. Effect of *lef-12* on early, late, and very late gene expression in SF-21 cells. The HSEpiHis *lef* library minus HSEpiHis *lef-12* was cotransfected with reporter plasmids containing the CAT gene under early (pETCAThr5) (lanes 1 and 2), late (pCAPCAT) (lanes 3 and 4), and very late (phcwt) (lanes 5 and 6) promoter control in the presence of HSEpiHis *lef-12* (+) (lanes 1, 3, and 5) or its frameshifted version (−) (lanes 2, 4, and 6). HSEpiHis *vlf-1* was added in lanes 5 and 6. The CAT activities shown below the lanes are relative to those of each of the reporter plasmids cotransfected with the HSEpiHis *lef* library and HSEpiHis *lef-12*. The acetylated chloramphenicol products (Ac Cm) and unacetylated substrate (Cm) are indicated on the right.

mids were unable to supply the functions of both of these genes is unknown. However, since each plasmid also contained frameshifted versions of either *p47* or *lef-12*, it is possible that one or both of the truncated *lef-12* or *p47* gene products exerted a dominant negative effect. The effect seemed to be limited to the context of SF-21 cells transfected with the Ac*M*NPV genomic library since transient assays using the HSE piHis *lef* library show clearly that *lef-12* is involved in baculovirus late gene expression. The fact that *lef-12* exerts little or no effect on transient late gene expression in the context of the genomic library is an additional reason why *lef-12* was not identified in the initial study of *lef* genes within p47* since these studies focused on finding *lef* genes within the context of the genomic library (51).

Through the constitutive expression of each LEF, we have determined that each LEF plays an independent role in expression of reporter genes under the transcriptional control of the late capsid promoter. Therefore, IE-1 and IE-2 appear to have roles in Ac*M*NPV late gene expression, in addition to their roles in transactivation of other *lef* genes. IE-1 and IE-2 influence the steady-state levels of plasmid DNA in a transient plasmid DNA replication assay (20, 28). Transient late gene expression and Ac*M*NPV late gene expression are both dependent on DNA replication, and therefore IE-1 and IE-2 may exert their apparent transregulatory effects through DNA replication. In vitro transcription experiments with nuclear extracts containing subsets of the HSEpiHis LEFs may uncouple the dependence of baculovirus transcription on DNA replication and help to resolve the roles of IE-1 and IE-2 in DNA replication and/or late gene transcription.

lef-12 has a role in late gene transcription and not virus DNA replication. p47* was not necessary for replication in transient assays using the *lef* library (28), and the addition of pHSEpiHis *lef-12* had no impact in assays using the HSEpiHis *lef* replica-

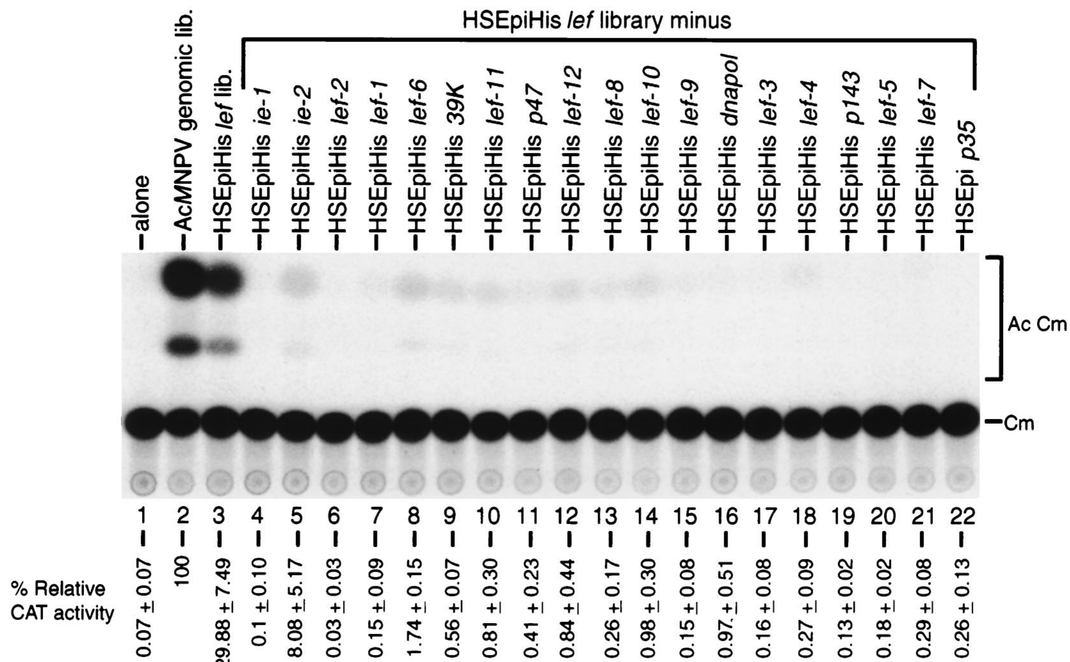

FIG. 6. Contribution of each HSEpiHis *lef* to late gene expression. Transient expression assays showing the levels of CAT activity from SF-21 cells transfected with the late reporter plasmid pCAPCAT alone (lane 1) or cotransfected with pCAPCAT and the Ac*M*NPV genomic library (lane 2), the HSEpiHis *lef* library (lane 3), or the HSEpiHis *lef* library lacking one of the 19 HSEpiHis *lef* genes (indicated above the lanes) (lanes 4 through 22). Cotransfections in lanes 4 through 22 each contained the empty vector pHSEpiHisVI$^+$ to equal the total amount of DNA in lane 3. The acetylated chloramphenicol products (Ac Cm) and unacetylated substrate (Cm) are indicated on the right. Relative CAT activities are shown below each lane.

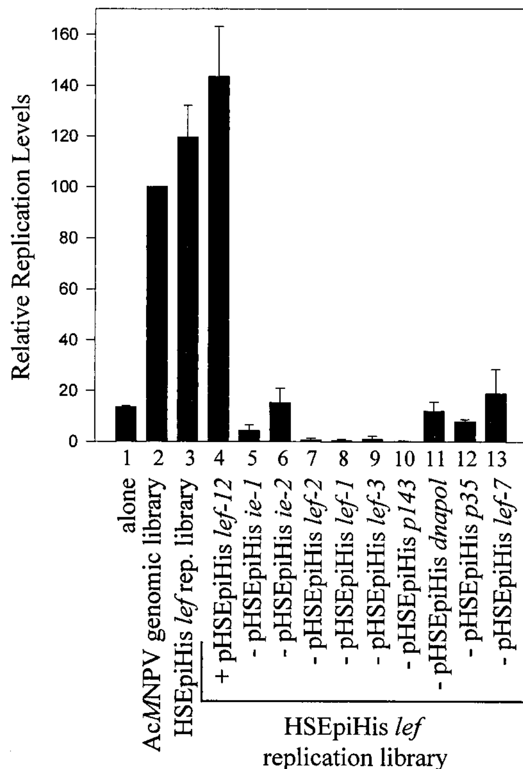

FIG. 7. Contribution of pHSEpiHis *lef-12* and each HSEpiHis replication *lef* to plasmid DNA replication. SF-21 cells were cotransfected with pCAPCAT (lane 1), pCAPCAT and the complete Ac*M*NPV genomic library (lane 2), pCAP CAT and the HSEpiHis *lef* replication library (lane 3), or pCAPCAT and the HSEpiHis *lef* replication library with the addition of pHSEpiHis *lef-12* or lacking one of the nine *lef* genes involved in plasmid replication (lanes 4 to 13). Levels of replicated plasmid were quantitated relative to those observed in the presence of the Ac*M*NPV genomic library. The results shown are representative of two independent experiments.

tion library. Therefore, like *p47*, *lef-12* is a transcription *lef*. *lef-12* is the only transcription-specific *lef* which demonstrates cell line specificity.

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