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Identification of Three Late Expression Factor Genes within the 33.8- to 43.4-Map-Unit Region of *Autographa californica* Nuclear Polyhedrosis Virus

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A transient transactivation assay system was used in combination with an overlapping *Autographa californica* nuclear polyhedrosis virus clone library to identify genes involved in late and very late baculovirus gene expression. We have identified three genes within the 33.8- to 43.4-map-unit region of the *A. californica* nuclear polyhedrosis virus genome which contribute to expression from promoters of the *vp39* major capsid protein and polyhedrin genes. One of these three genes corresponds to the previously identified DNA polymerase gene, while the other two genes encode previously unidentified polypeptides of 59,418 and 8,706 Da. None of these genes were required for expression from the early *etl* promoter.

Autographa californica nuclear polyhedrosis virus (Ac*M*NPV) serves as the primary model system for the study of gene function and gene regulation in the *Baculoviridae* family. Ac*M*NPV genes are expressed in a coordinately regulated and sequentially ordered fashion (see reference 26 for a review). During infection, three main transcriptional phases are recognized: early, late, and very late. Early genes are apparently transcribed by host RNA polymerase II (9, 14, 15) and are expressed in the absence of prior viral protein synthesis, whereas late and very late genes are transcribed by an alpha-amanitin-resistant RNA polymerase activity (11, 15). Late gene transcription depends on viral DNA replication (8, 10, 36).

Three genes which appear to be associated with DNA replication have been identified in the Ac*M*NPV genome. These genes, *etl* (7, 25), *dnapol* (38), and *p143* (21), are homologs of proliferating cell nuclear antigen, DNA polymerase, and DNA helicase genes, respectively. DNA replication and late gene expression are delayed in an Ac*M*NPV insertion mutant carrying a *lacZ* insertion in *etl* (7, 25) and are inhibited at the nonpermissive temperature during infection with a temperature-sensitive mutant with a mutation in *p143* (10, 21). The involvement of *dnapol* in DNA replication has been indicated by transient plasmid DNA replication assays (17a), but its involvement in late gene expression has not yet been investigated.

Our understanding of the genes involved in the progression from early to late gene expression has been greatly facilitated by the development of a method to identify viral genes that transactivate reporter genes under late viral promoter control, by using an overlapping library of Ac*M*NPV clones to transactivate expression from a reporter plasmid (27). *Spodoptera frugiperda* cells which have been transfected with a complete overlapping library of Ac*M*NPV clones are able to support significant levels of expression from the late *vp39* capsid protein promoter. By omission of individual or overlapping library clones containing one or more transactivating genes and subsequent substitution of clones by subclones, 11 late expression factor genes (*lef*s) have been identified (20, 22, 27–31). These 11 genes also have been shown to be involved in expression from the very late *polh* promoter.

Some of the genes identified by this assay may not be involved directly in late gene transcription or translation. Two of these genes, *ie-1* and *ie-n*, are *trans* regulators of early gene expression (3, 5, 6, 12, 13, 23). The DNA helicase gene homolog, *p143*, is known to be required for DNA replication but is also required for optimum expression from the late *vp39* and very late *polh* promoters in this assay (29). Of the remaining eight *lef*s, some probably participate in viral DNA replication, while others are likely to be associated with the novel alpha-amanitin-resistant RNA polymerase complex. Consistent with this idea is the identification of a highly conserved RNA polymerase motif within the 102-kDa polypeptide encoded by *lef-8* (31).

In this paper we report the identification of three *lef*s within the 33.8- to 43.4-map-unit (m.u.) region of the Ac*M*NPV genome. One of these *lef*s is *dnapol*, while the remaining two *lef*s (*lef-9* and *lef-10*) are genes which have been identified by sequence only.

MATERIALS AND METHODS

Cells and virus. Ac*M*NPV L-1 (18) was propagated in the IPLB-SF-21 (SF-21) cell line (39). 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% typtose broth (26).

Plasmid constructs. The early (pETCAThr5), late (pCAP-CAT), and very late (phcwtCAT) promoter-chloramphenicol acetyltransferase (CAT) reporter plasmids were previously described (24, 27, 37). Plasmid contructs pRI-M and pSDEM2, containing *lef-8* and *lef-3*, respectively, were previously described (20, 31). The plasmid pPstHI contains the identical Ac*M*NPV region (33.8 to 43.4 m.u.) found in the lambda PstHI clone (27). pPstHI was constructed by digesting lambda PstHI with *Sfi*I, which has recognition sites flanking the viral DNA insert, repairing the ends with the *Escherichia coli* DNA polymerase I Klenow fragment (GIBCO BRL), and inserting

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this DNA into the Klenow-repaired *Xba*I site of pBluescriptKS(+).

(i) Subclones of pPstHI. The pPstHI deletion clones (Fig. 1A) pPstHIXhoI, pPstHIHindIII, and pPstHISmaI were constructed by digestion of pPstHI with *Xho*I, *Hin*dIII, and *Sma*I, respectively, followed by religation to the *Xho*I, *Hin*dIII, or *Sma*I sites within the multicloning site of pBluescriptKS(+). pPstHIXhoI contains a deletion between 33.8 and 34.7 m.u., pPstHIHindIII contains a deletion between 33.8 and 37.3 m.u., and pPstHISmaI contains a deletion between 33.8 and 40.3 m.u. pPstHISstI was constructed by digesting pPstHI with *Sst*I, isolating the 5.1-kb fragment, and inserting this fragment into the *Sst*I site of pBluescriptKS(+). The resulting plasmid represented a pPstHI subclone deleted between 33.8 and 39.6 m.u.

pPstHIHStu3.3 contains a 3.3-kb *Hin*dIII (33.8 m.u.)-*Stu*I (36.1 m.u.) fragment generated by digestion of pPstHI with *Hin*dIII (within the multicloning site) and *Stu*I and subsequent insertion of this fragment into the *Hin*dIII and *Sma*I sites of pBluescriptKS(+). pPstHIEB4.7 was constructed by digestion of pPstHI with *Eco*RI and *Bgl*II. A 4.7-kb fragment representing 34.8 to 38 m.u. was isolated and cloned into the *Eco*RI and *Bam*HI sites of pBluescriptKS(+). pDNAp was constructed by complete digestion of lambda PstHI with *Not*I followed by partial digestion with *Eco*RI. A 4.2-kb fragment (39.5 to 43.4 m.u.) containing the intact *dnapol* gene was isolated and cloned into pBluescriptSK(+) between the *Not*I and *Eco*RI sites. pDNApfs was constructed by partial *Pst*I digestion of pDNAp, repair of the ends with T4 DNA polymerase (GIBCO BRL), and subsequent religation of the plasmid. The resulting plasmid, pDNApfs, contains a frameshift mutation at the *Pst*I site at 42.0 m.u. that results in the premature termination of DNA polymerase synthesis after 57 amino acid residues. Sequence analysis was used to confirm that a frameshift mutation was generated at the *Pst*I site.

(ii) Subclones of the 34.8- to 38.0-m.u. region. pPstHIES1.3 was constructed by digestion of a subclone of pPstHI, pPst HIHS7.7, which contains a 7.7-kb *Sst*I fragment corresponding to 33.8 to 39.6 m.u. on the Ac*M*NPV genome. pPstHIHS7.7 was digested with *Eco*RI and *Sal*I to generate a 1.3-kb fragment, representing 34.8 to 35.5 m.u., which was isolated and inserted into the corresponding sites in pBluescriptKS(+). pPstHIEA2.4 was made by subcloning from pPstHIEB4.7 a 2.4-kb *Apa*I fragment corresponding to 34.8 to 36.4 m.u. into the *Apa*I site of pBluescriptKS(+). pPstHIStuB2.8 is derived from pPstHIEB4.7 and contains a 2.8-kb *Stu*I-*Bgl*II fragment representing 36.1 to 38.0 m.u. on the Ac*M*NPV genome. pPstHISB2.35 contains a 2.35-kb *Sal*I (36.5 m.u.)-*Bgl*II (38.0 m.u.) fragment. pPstHISB2.35fsHindIII is a clone identical to pPstHISB2.35 except that it contains a frameshift mutation at the *Hin*dIII site at 37.3 m.u. This mutation was created by digestion of pPstHISB2.35 with *Hin*dIII followed by repair of the ends with the Klenow fragment prior to the religation of the plasmid.

(iii) Subclones of the 33.8- to 35.9-m.u. region. pPstHISal2.0 contains a 2.0-kb *Sal*I fragment representing 34.3 to 35.9 m.u. on the Ac*M*NPV genome. pPstHIEco1.4 is a subclone of pPstHIHStu3.3 containing a 1.4-kb *Eco*RI fragment extending from 33.8 to 34.8 m.u. pPstHISal0.65 was subcloned from pPstHIEco1.4 by digesting pPstHIEco1.4 with *Sal*I and inserting a 0.65-kb fragment (33.8 to 34.2 m.u.) into the *Sal*I site of pBluescriptKS(+). The plasmids pPstHIHc0.25 and pPstHIHc 0.4 were constructed by digesting pPstHISal0.65 with *Hin*cII and inserting either a 0.25-kb *Hin*cII fragment (33.8 to 34.0 m.u.) or a 0.4-kb *Hin*cII fragment (34.0 to 34.2 m.u.) into pBluescriptKS(+) at the *Hin*cII site. The plasmids pPstHIME 0.5 and pPstHIEM0.4 were constructed by complete digestion of pPstHISal0.65 with *Eco*RI (in the vector) followed by partial digestion with *Mlu*I. Fragments of 0.4 and 0.5 kb were isolated and cloned into the *Sma*I site of pBluescriptKS(+) after the ends were repaired by the Klenow fragment. pPstHIME0.5 and pPstHIEM0.4 contain fragments corresponding to about 33.96 to 34.2 m.u. and 33.8 to 34.07 m.u., respectively. pPstHIMF0.26 was constructed by deleting a 0.24-kb *Fok*I-*Eco*RI fragment from pPstHIME0.5, resulting in a plasmid with a 0.26-kb insert representing 33.9 to 34.05 m.u. on the Ac*M*NPV genome. pPstHIEM0.5fs is identical to pPstHIEM0.5 except that it contains a frameshift mutation at the *Fok*I site generated by partial digestion of pPstHIEM0.5 with *Fok*I, repair of the ends by the Klenow fragment, and subsequent religation of the ends. The sequence at the frameshift was verified by sequence analysis.

RESULTS

At least three different ORFs, including *dnapol*, within the region from 33.8 to 43.4 m.u. are involved in late gene expression. The *dnapol* gene is located within the lambda PstHI clone (33.8 to 43.4 m.u.) of the overlapping Ac*M*NPV clone library which transactivates late reporter gene expression in transient expression assays (27). In addition to *dnapol*, this clone contains two previously identified genes, *fp25* (2) and *p34.8* (40). The lambda PstHI clone is overlapped by lambda clones ETL7 (20.1 to 34.8 m.u.), PstH4 (25.0 to 42.1 m.u.), and PstH5 (34.8 to 50.3 m.u.) (Fig. 1A). Two known *lef*s, *lef-3* and *lef-8*, are located in the regions immediately flanking lambda PstHI. *lef-3* is located between 43.4 and 45.2 m.u. within a unique region of lambda PstH5 (20), and *lef-8* is located between 30.1 and 32.9 m.u. within the double overlap between lambda clones ETL7 and PstH4 (31).

To determine whether *dnapol* contributed to late gene expression in the transient expression assay, lambda clones PstH4, PstHI, and PstH5 were omitted from the Ac*M*NPV clone library and a plasmid (pSDEM2) was added to supply *lef-3* function. Similar levels of CAT expression from reporter plasmid pCAPCAT were observed when *lef-3* and pPstHI, a plasmid version of the original lambda PstHI clone, were used in the absence of lambda PstH4 and PstH5 (Fig. 1B; compare lanes 2 and 3). Omission of pPstHI from this group of clones reduced expression levels to the background level (Fig. 1B; compare lanes 1 and 4), indicating that a gene(s) within PstHI was required for expression from the late *vp39* promoter in pCAPCAT.

Selected restriction enzymes were used to digest pPstHI in order to determine if specific regions of PstHI might be involved in transactivating reporter gene expression. pPstHI DNAs digested with *Xho*I, *Eco*RI, *Hin*dIII, *Pst*I, *Bgl*II, *Sst*I, or *Sma*I were individually used in place of intact pPstHI in transient expression assays that included the Ac*M*NPV clone library without lambda PstH4 and PstH5 but supplemented with *lef-3* (Fig. 1B, lanes 5 to 11). Digestion of pPstHI by *Xho*I, *Bgl*II, or *Sst*I did not affect late gene expression (Fig. 1B, lanes 5, 9, and 10, respectively), indicating that these sites did not disrupt a *lef*. Thus, *p34.8* (40), the only open reading frame (ORF) within PstHI disrupted by digestion with *Sst*I, was not involved in late gene expression. Expression from pCAPCAT was reduced 15- to 20-fold, however, by digestion of pPstHI with *Eco*RI, *Hin*dIII, *Pst*I, and *Sma*I (Fig. 1B, lanes 6, 7, 8, and 11, respectively). The *Sma*I site is unique to *dnapol*, which strongly implicates this gene in late gene expression. A plasmid, pDNAp, containing *dnapol* and limited flanking regions could not alone substitute for pPstHI in the assay (Fig. 1B, lane

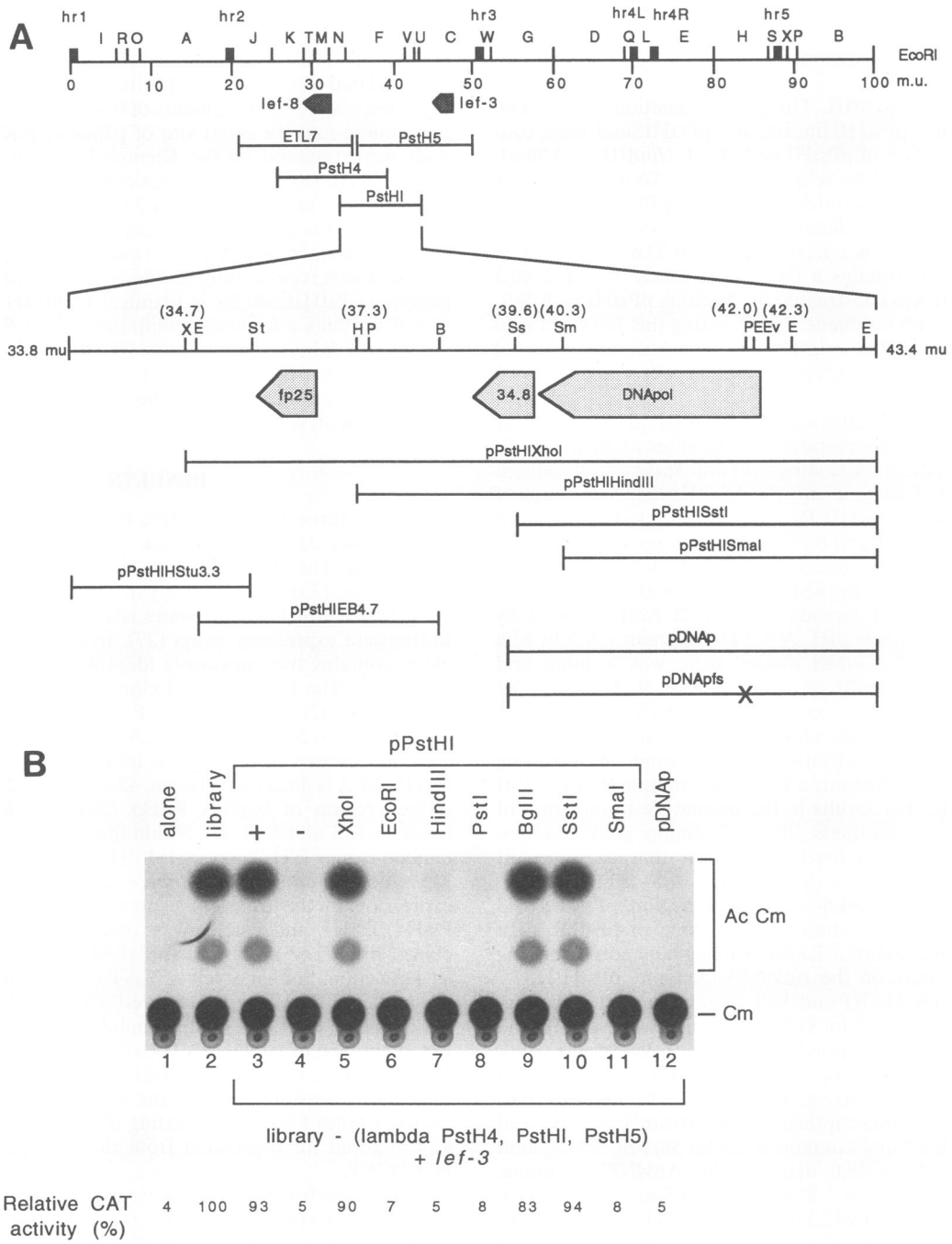


FIG. 1. Identification of regions within the 33.8- to 45.4-m.u. region of the Ac*M*NPV genome required for late gene expression. (A) The locations of the lambda library clones overlapping the 33.8- to 45.4-m.u. region are shown below an *Eco*RI restriction map of a linearized representation of the Ac*M*NPV genome. The approximate locations of *lef-3* and *lef-8* are also indicated. The region contained within the lambda PstHI clone is illustrated below, along with the locations and orientations of the previously described genes *dnapol* (38), *p34.8* (40), and *fp25* (2). The locations of deletion clones and subclones of pPstHI used in the transient expression assays are also indicated. Restriction sites are abbreviated as follows: X, *Xho*I; E, *Eco*RI; Ev, *Eco*RV; St, *Stu*I; H, *Hin*dIII; P, *Pst*I, B, *Bgl*II; Ss, *Sst*I; and Sm, *Sma*I. Not all *Stu*I sites are indicated. (B) CAT activities of cells transfected with pCAPCAT alone (lane 1), pCAPCAT and the complete Ac*M*NPV clone library (lane 2), or pCAPCAT and a partial library supplemented (lane 3) or not supplemented (lane 4) with intact pPstHI or supplemented with pPstHI digested with the indicated restriction enzymes (lanes 5 to 11). Library clones omitted from the complete library and their substitutions are shown below the lanes. A plasmid, pSDEM2 (2), was used to supply *lef-3*. The effect of addition of a plasmid (pDNAp) containing an intact *dnapol* gene is shown in lane 12. CAT activities relative to that of pCAPCAT in the presence of the complete library (lane 2) are indicated at the bottom. (C) CAT activities of cells transfected with pCAPCAT alone (lane 1) or pCAPCAT in the presence of either the entire Ac*M*NPV library (lane 2) or a partial clone library (lanes 3 to 13). DNAs omitted from or added to the complete library are indicated below the lanes. In these assays the plasmids pSDEM2 and pRI-M were used to supply *lef-3* and *lef-8*, respectively. Additional plasmids used to supplement the partial library are indicated above each lane. CAT activity relative to that of pCAPCAT with the complete library is shown below each lane. For quantitation, cell extracts were diluted so that less than 40% of the chloramphenicol was acetylated. The thin-layer chromatography and CAT activity data are representative of two independent experiments. A difference of less than twofold was not considered significant. The positions of acetylated (Ac Cm) and unacetylated (Cm) chloramphenicol are shown at the right.

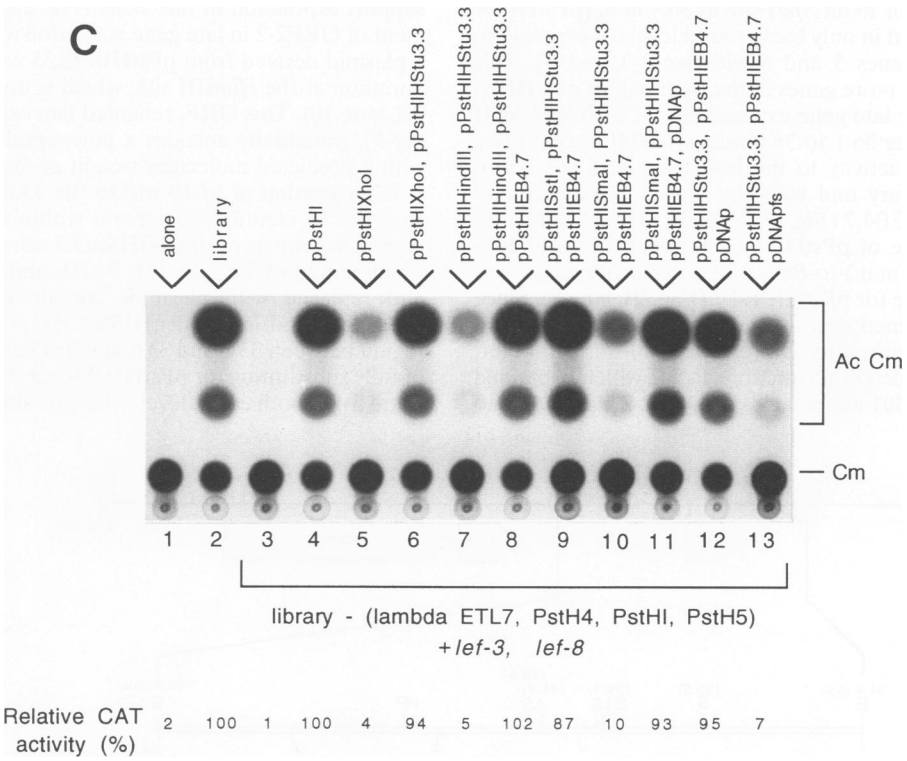


FIG. 1—*Continued.*

12), indicating that additional *lef*s were being supplied by pPstHI. Since digestion at the unique *Hin*dIII site (37.3 m.u.) upstream of *fp25* also eliminated late expression, a gene located in this region of pPstHI is also required for late reporter gene expression.

In order to further define regions within PstHI that supplied *lef* functions, a series of pPstHI deletion clones was constructed and used to replace pPstHI in a partial Ac*M*NPV clone library in which lambda clones ETL7, PstH4, PstHI, and PstH5 were omitted and pPstHI, *lef-3*, and a plasmid containing *lef-8* (pRI-M) were added. This combination of plasmids provided expression equivalent to that of the original Ac*M* NPV library (Fig. 1C; compare lanes 2 and 4) and allowed the examination of the entire pPstHI region for the presence of *lef*s. Substitution of pPstHI with pPstHIXhoI, in which the 33.8- to 34.7-m.u. region of pPstHI was deleted, reduced expression 25-fold (Fig. 1C; compare lanes 4 and 5). Addition of a plasmid which overlapped this region (pPstHIHStu3.3) restored almost full expression (Fig. 1C, compare lanes 2, 4, and 6). This suggested that an additional *lef* was located within the 33.8- to 34.7-m.u. region of the Ac*M*NPV genome.

Late reporter gene expression was also significantly decreased (about 20-fold) when pPstHI was replaced by pPstHIH Stu3.3 and a plasmid containing a deletion between 33.8 and 37.3 m.u., pPstHIHindIII (Fig. 1C; compare lanes 6 and 7). Expression was restored to library levels if, in addition to pPstHIHStu3.3 and pPstHIHindIII, another plasmid containing the omitted region, pPstHIEB4.7, was included in the assay (Fig. 1C, lane 8). The presence of a third *lef* in this region of pPstHI was consistent with the observation that *Hin*dIII digestion of pPstHI resulted in reduced expression (Fig. 1B, lane 6).

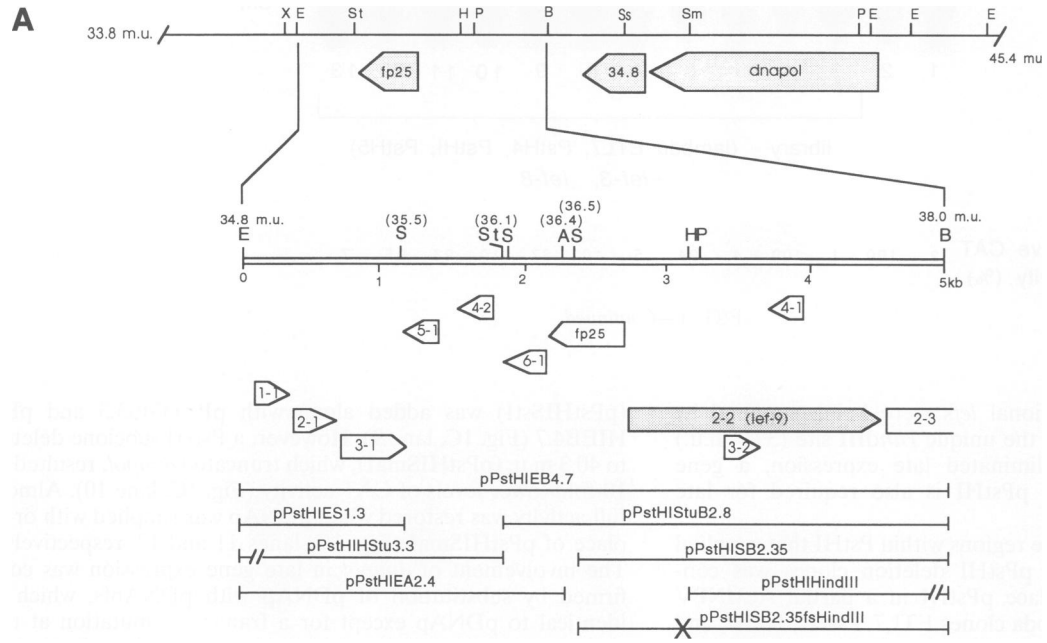
Late gene expression was not dramatically affected when a subclone of PstHI containing only the 39.6- to 45.4-m.u. region (pPstHISstI) was added along with pPstHStu3.3 and pPst HIEB4.7 (Fig. 1C, lane 9). However, a PstHI subclone deleted to 40.3 m.u. (pPstHISmaI), which truncated *dnapol*, resulted in 10-fold-lower levels of CAT activity (Fig. 1C, lane 10). Almost full activity was restored when pDNAp was supplied with or in place of pPstHISmaI (Fig. 1C, lanes 11 and 12, respectively). The involvement of *dnapol* in late gene expression was confirmed by substitution of pDNAp with pDNApfs, which is identical to pDNAp except for a frameshift mutation at the *Pst*I site (42.0 m.u.) (Fig. 1C, lane 13). The resulting frameshift is expected to delete 927 amino acids from the C terminus of the DNA polymerase. Another plasmid, pDNApEcoRV, containing a deletion in pDNAp between 42.3 and 43.4 m.u. upstream of *dnapol*, was also capable of supporting full expression from pCAPCAT (data not shown). Thus, the only *lef* provided by pDNAp is *dnapol* unless smaller ORFs contained within *dnapol* also contribute to late gene expression.

Identification of *lef-9* within the 34.8- to 38.0-m.u. region. The results of the pPstHI deletion analysis suggested that at least two genes in addition to *dnapol* were necessary for expression from the reporter plasmid pCAPCAT. One of these genes was supplied by pPstHIEB4.7, which contains the 34.8- to 38.0-m.u. region of Ac*M*NPV. Several ORFs are located within this region, including *fp25* (Fig. 2A [the nucleotide sequence data were obtained from reference 1]). Subclones of this region were used to substitute for pPstHIEB4.7 in transient expression assays. These assays were performed with a partial Ac*M*NPV clone library containing, in place of the lambda clones ETL7, PstH4, PstHI, and PstH5, plasmids encoding *lef-8*, *lef-3*, and *dnapol*, as well as pPstHIHStu3.3, which supplied at least one *lef* (see above).

Substitution of pPstHIEB4.7 with plasmids containing the region from the *Eco*RI site at 34.8 m.u. to a *Sal*I site at 35.5

m.u. (pPstHIES1.3) or to an *Apa*I site at 36.4 m.u. (pPstHIEA 2.4) (Fig. 2A) resulted in only background levels of expression. (Fig. 2B; compare lanes 5 and 6 with lanes 1 and 3). This indicated that one or more genes in the right half of pPstHIEB 4.7 were required for late gene expression. A 2.8-kb *Stu*I-*Bgl*II fragment representing 36.1 to 38.0 m.u. (pPstHIStuB2.8) was able to reconstitute activity to the level seen with the entire Ac*M*NPV clone library and with the partial library supplemented with pPstHIEB4.7 (Fig. 2B; compare lane 7 with lanes 2 and 4). A subclone of pPstHIStuB2.8, pPstHISB2.35, containing a *Sal*I (36.5 m.u.)-to-*Bgl*II (38.0 m.u.) fragment, was also able to substitute for pPstHIEB4.7 (Fig. 2B, lane 8). Since pPstHISB2.35 contained only a truncated *fp25* gene, *fp25* did not appear to be required for expression from this promoter. The pPstHIHindIII deletion clone of pPstHI which contained only the C-terminal 301 amino acids of ORF2-2 was unable to support expression in this assay (Fig. 2B, lane 9) The involvement of ORF2-2 in late gene activation was confirmed by using a plasmid derived from pPstHISB2.35 containing a frameshift mutation at the *Hin*dIII site, which is unique to ORF2-2 (Fig. 2B, lane 10). This ORF, renamed late expression factor gene 9 (*lef-9*), potentially encodes a polypeptide of 516 amino acids with a predicted molecular weight of 59,418 Da (1).

Identification of *lef-10* within the 33.8- to 35.9-m.u. region. In order to identify *lef*s present within the 33.8- to 35.9-m.u. region, subclones of pPstHIHStu3.3 were added to a library in which lambda ETL7, PstH4, PstHI, and PstH5 were removed and replaced with plasmids containing *lef-3*, *lef-8*, *dnapol* (pDNAp), and *lef-9* (pPstHISB2.35). A clone containing the region between 33.8 and 34.8 m.u. (pPstHIEco1.4) was capable of fully substituting for pPstHIHStu3.3 (Fig. 3B; compare lanes 4 and 5). In both cases, levels of expression were comparable to



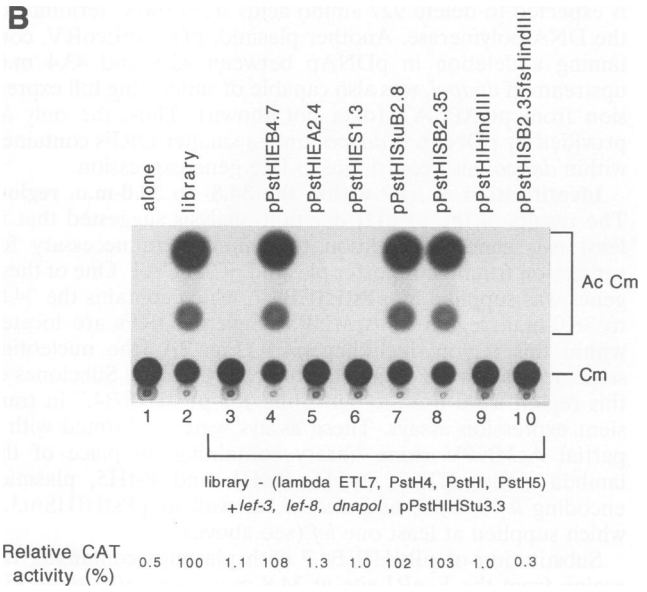


FIG. 2. Identification of *lef-9*. (A) Restriction map of the 33.8- to 45.4-m.u. region of Ac*M*NPV with the orientations and locations of known genes found in this region. Below this map is an enlargement of the 34.8- to 38.0-m.u. region containing *lef-9*, showing a restriction map and the distribution of ORFs larger than 50 amino acids (arrow boxes). ORFs are designated according to their reading frame (first number) and by their order in that frame (second number). Subclones that were used to locate *lef-9* (shaded arrow box) are indicated below. A frameshift mutation introduced into *lef-9* is designated by an X. Abbreviations for restriction enzymes: X, *Xho*I; E, *Eco*RI; St, *Stu*I; H, *Hin*dIII; P, *Pst*I; B, *Bgl*II; Ss, *Sst*I; Sm, *Sma*I; S, *Sal*I; and A, *Apa*I. (B) CAT activities of cell lysates prepared from cells transfected with pCAPCAT alone (lane 1) or with pCAPCAT and the complete Ac*M*NPV clone library (lane 2) or a partial clone library (lanes 3 to 10). The specific library clones omitted in the partial library and their substitutions are indicated below the lanes. The plasmids pRI-M, pSDEM2, and pDNAp were used to supply *lef-8*, *lef-3*, and *dnapol*, respectively. Additional plasmids added to the partial library are shown above each lane. CAT activities relative to that for lane 2 are presented at the bottom (see legend to Fig. 1 for details).

that observed with the entire Ac*M*NPV clone library (Fig. 3B, lane 2). A 2.0-kb *Sal*I fragment (34.3 to 35.9 m.u.) which partially overlapped pPstHIEco1.4 did not significantly increase expression above background levels (Fig. 3B; compare lanes 1, 3, and 6). Thus, the 1.4-kb *Eco*RI fragment contained a gene(s) involved in late gene expression, and ORFs 1-1, 2-1, 3-1, 5-1, and 4-2, located between 34.8 and 36.1 m.u. (Fig. 2A), were not required for late reporter gene expression.

The 1.4-kb region between 33.8 and 34.8 m.u. contains three complete ORFs (6-1, 2-1, and 4-1) and two incomplete ORFs (3-1 and 3-2) larger than 50 amino acids (Fig. 3A [the nucleotide sequence data were obtained from reference 1]). In order to determine which of these ORFs was contributing to late gene expression, subclones of pPstHIEco1.4 were tested in the transient assay. Levels of expression equivalent to that seen with pPstHIEco1.4 were observed when a 0.65-kb *Sal*I fragment (pPstHISal0.65) corresponding to 33.8 to 34.2 m.u. was used (Fig. 3B; compare lanes 5 and 7). Thus, ORF4-1 was eliminated as a *lef* candidate, and ORF3-2, which is truncated by more than half its length, is unlikely to contribute in the assay. The pPstHISal0.65 contained only two full-length ORFs, 6-1 and 2-1. Expression was not restored by addition of pPstHIHc0.25, a subclone of pPstHISal0.65 containing only ORF6-1. Addition of pPstHIHc0.4, which encodes a truncated ORF2-1 that is missing its first 11 amino acids, provided approximately 20% of the activity provided by the library (Fig. 3B, lanes 8 and 9). This suggested that ORF2-1 was involved in late gene expression; however, it was also possible that pPst HIHc0.25 did not reconstitute activity because ORFs 6-1 and 2-1 each contribute a factor providing optimal expression.

To investigate the possibility that both ORFs 6-1 and 2-1 are *lef*s, two subclones of pPstHISal0.65 were constructed: pPst HIME0.5 contained a complete ORF2-1 and an ORF6-1 truncated by 28 codons, while pPstHIEM0.4 contained a complete ORF6-1 and an ORF2-1 truncated by 3 codons. Each construct was tested for its ability to replace pPstHISal0.65 in transient expression assays. Both plasmids were able to fully substitute for pPstHISal0.65 (Fig. 3B, lanes 10 and 11). Only 16 of the 45 amino acid residues specified by ORF6-1 remained in pPstHIME0.5, indicating that ORF6-1 probably did not play a role in late reporter gene expression. Since pPstHIEM0.4 truncated ORF2-1 by only three codons, it was likely that the product of ORF2-1 was still functional in our assay. We therefore constructed a subclone of pPstHIME0.5 which deleted 15 amino acids from the ORF2-1 C terminus (pPstHIMF 0.26) and a subclone of pPstHIME0.5, pPstHIME0.5fs, containing a frameshift mutation at the same *Fok*I site used to truncate ORF2-1 in pPstHIMF0.26. In transient expression assays, both plasmids behaved similarly, reducing CAT expression moderately but significantly (Fig. 3B, lanes 12 and 13). A frameshift mutation introduced at a *Not*I site 32 bp upstream of the *Fok*I site reduced CAT expression levels 10-fold, confirming that ORF2-1 contributed to expression from pCAPCAT (data not shown). This ORF was named late expression factor gene 10 (*lef-10*) and potentially encodes a 79-amino-acid polypeptide with a predicted molecular weight of 8,706 Da, the smallest LEF identified to date.

***lef-9*, *lef-10*, and *dnapol* are required for late and very late but not early gene expression.** The contributions of *lef-9*, *lef-10*, and *dnapol* to early and very late gene expression were tested by using CAT reporter plasmids under the control of the *etl* promoter (pETCAThr5) and the *polh* promoter (phcwtCAT), respectively. pETCAThr5, pCAPCAT, and phcwtCAT were cotransfected with a partial Ac*M*NPV clone library in which lambda clones ETL7, PstH4, PstHI, and PstH5 were replaced with plasmid clones containing *lef-8*, *lef-10*, *lef-9*, *dnapol*, and *lef-3*. In the case of the pETCAThr5 reporter plasmid, little or no decrease in CAT expression was observed when *lef-10*, *lef-9*, or *dnapol* was replaced by a plasmid containing a frameshift mutation within that gene (Fig. 4; compare lanes 2, 3, and 4 with lane 1). The effects of these *lef*s on expression from this early promoter were determined 24 h posttransfection so that the effects of plasmid replication would be minimal (19). As expected, expression from pCAPCAT was significantly reduced if any of these genes were replaced by their corresponding frameshifted genes (Fig. 4; compare lanes 6, 7, and 8 with lane 5). In addition, these genes were also required for substantial expression from the *polh* promoter of the phcwt-CAT reporter plasmid (Fig. 4; compare lanes 10, 11, and 12 with lane 9). These results confirm that *lef-10*, *lef-9*, and *dnapol* are late expression factor genes which influence both late and very late gene expression but not early gene expression.

DISCUSSION

We have identified three more late expression factor genes in the Ac*M*NPV genome. These *lef*s, located between 33.8 and 43.4 m.u., are involved in expression of reporter genes under the transcriptional control of the late capsid and very late polyhedrin promoters.

No significant extensive homologies to other genes within the nucleic acid and protein databases have been found for either *lef-9* or *lef-10*. However, the derived amino acid sequence of *lef-9* does contain an amino acid motif (NTDC DGD) similar in five of seven positions to a highly conserved motif (NADFDGD) found in the largest subunits of a number of DNA-directed RNA polymerases (4, 16).

Our results show that *lef-9*, *lef-10*, and *dnapol*, as well as all the other *lef*s found to date, are necessary for efficient late and very late gene expression in a transient assay system. Whether these genes are essential in vivo remains to be determined. Two genes identified by transient assays, *ie-1* and *p143*, are known to be essential in the context of the virus from investigations using temperature-sensitive mutants (21, 35).

The identification of *dnapol* as a late expression factor gene is not surprising given the apparent dependence of late and very late gene expression on DNA replication. The recent finding that the *Orgyia pseudotsugata* nuclear polyhedrosis virus DNA polymerase gene is necessary in a transient replication assay system (33) is consistent with this interpretation. Therefore, this assay may accurately reflect the events occurring during virus infection with respect to the requirement for DNA replication prior to or concomitant with the expression of late genes. The reporter plasmids used in our assays contain an homologous repeat sequence; homologous repeat regions appear to function as *cis*-acting origins of DNA replication for transfected plasmid DNAs (17, 19, 32). Previous studies, however, have demonstrated that the presence or absence of homologous repeat regions does not significantly affect expression from either pCAPCAT or phcwtCAT (34, 35) in transient assays. How these observations relate to our results that *dnapol*, and thus presumably DNA replication, is required for late gene expression in this assay system will need to be resolved once all the *lef*s and replicative genes have been identified. We are currently determining whether the *lef*s function at the level of DNA replication, transcription, or translation.

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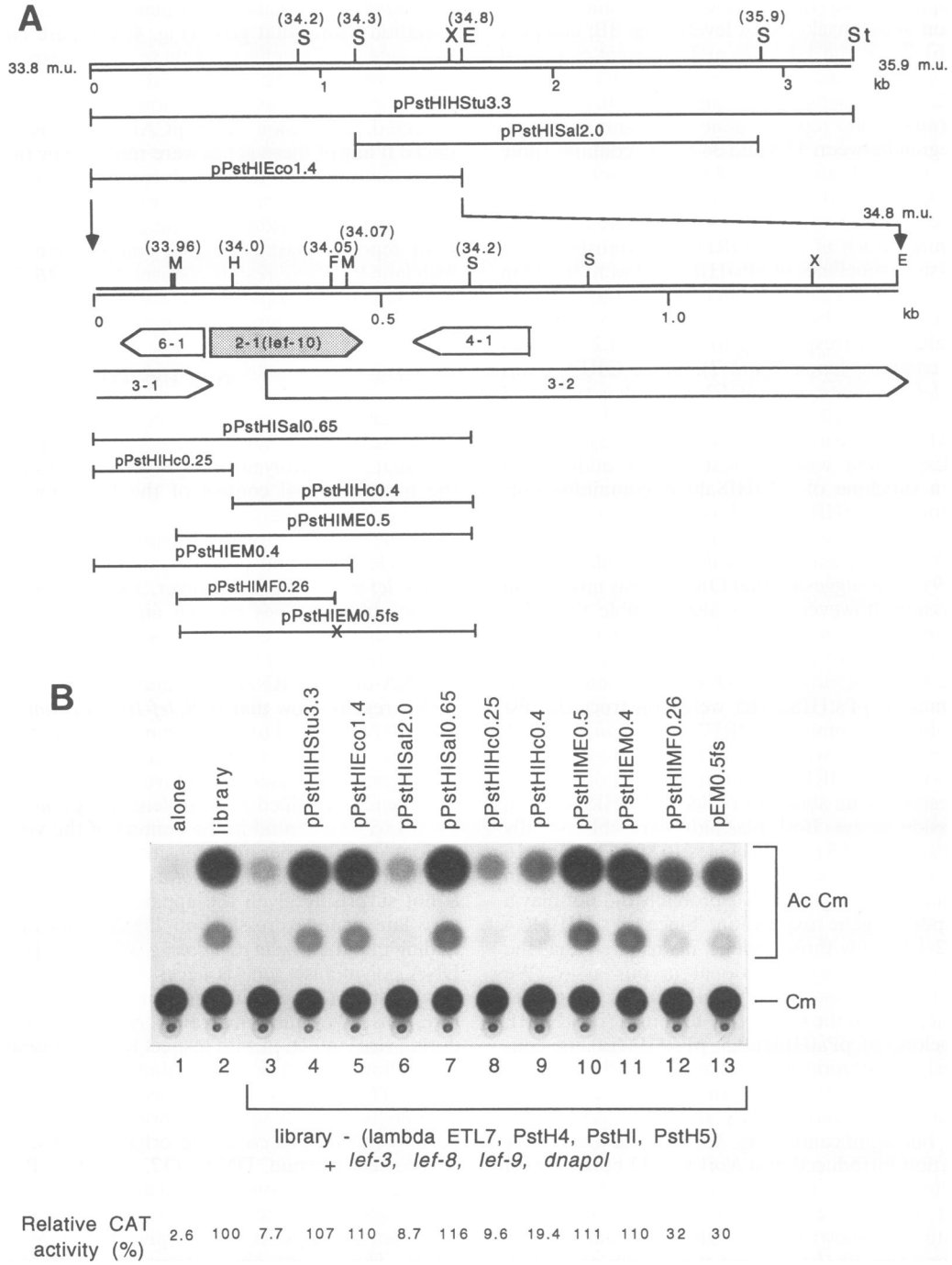

FIG. 3. Identification of *lef-10*. (A) Restriction map of the 33.8- to 35.9-m.u. region of Ac*M*NPV along with the subclones from this region used in transient expression assays to locate *lef-10*. An expanded portion showing the locations and orientations of ORFs (arrow boxes) larger than 50 amino acids relative to a restriction map of the 33.8- to 34.8-m.u. region is presented below. Also shown are the positions of subclones used to identify *lef-10* (shaded arrow box). The location of a frameshift mutation in *lef-10* is designated by an X. Restriction enzyme abbreviations: S, *Sal*I; X, *Xho*I; E, *Eco*RI; St, *Stu*I; M, *Mlu*I; Hc, *Hin*cII; and F, *Fok*I. Not all *Mlu*I and *Hin*cII sites are shown. (B) CAT activities in the absence (lane 1) or presence (lane 2) of the complete Ac*M*NPV clone library and in the presence of a partial library (lanes 3 to 13). Library clones omitted from the complete library and additional *lef*-containing plasmids included in the partial library are shown below the lanes. The plasmids pSDEM2, pRI-M, pDNAp, and pPstHISB2.35 were used to supply *lef-3*, *lef-8*, *dnapol*, and *lef-9*, respectively, to the partial library. Individual subclones of the 33.8- to 35.9-m.u. region added to the partial library are shown above each lane. CAT activities relative to that of pCAPCAT in the presence of the complete library (lane 2) appear at the bottom (see legend to Fig. 1 for details).

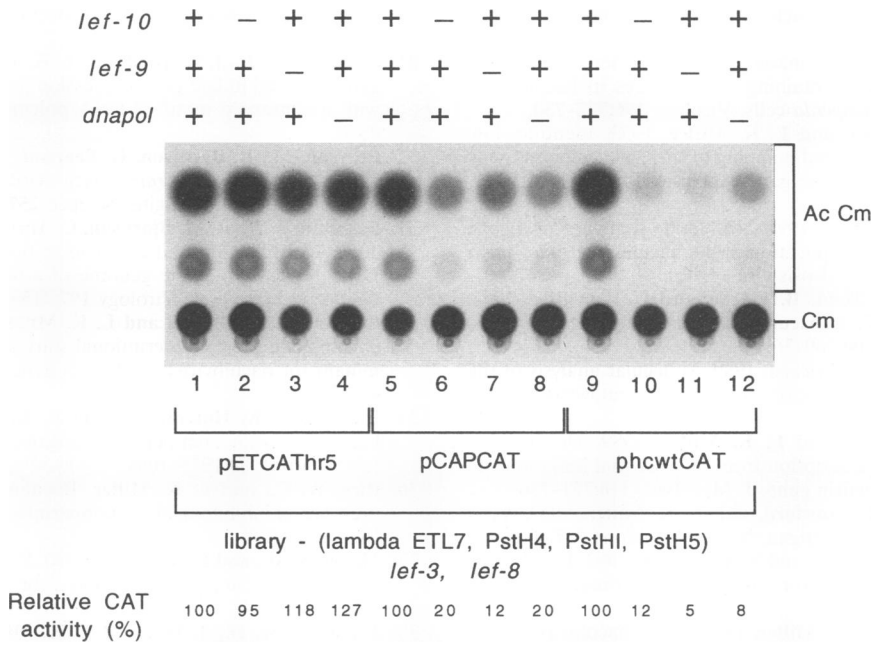

FIG. 4. Requirements for *lef-9*, *lef-10*, and *dnapol* in early (pETCAThr5), late (pCAPCAT), and very late (phcwtCAT) gene expression. CAT activities of pETCAThr5 (lanes 1 to 4), pCAPCAT (lanes 5 to 8), and phcwtCAT (lanes 9 to 12) in the presence of a partial library in which lambda ETL7, PstH4, PstHI, and PstH5 were omitted and plasmids containing *lef-8* (pRI-M) and *lef-3* (pSDEM2) were added are shown. Cells transfected with pETCAThr5, pCAPCAT, and phcwtCAT were harvested at 24, 48, and 72 h posttransfection, respectively. The addition of plasmids containing *lef-9* (pPstHISB2.35), *lef-10* (pPstHIME0.5), and *dnapol* (pDNAp) or their corresponding frameshifts are indicated above each lane as + or −, respectively. CAT activities within each promoter group (see legend to Fig. 1 for details) are relative to that of the promoter in the presence of *lef-9*, *lef-10*, and *dnapol* (lanes 1, 5, and 9, respectively).

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