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Three Baculovirus Genes Involved in Late and Very Late Gene Expression: *ie-1*, *ie-n*, and *lef-2*

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We developed a method to identify baculovirus genes required for late and very late gene expression that is based on subtraction of clones from an *Autographa californica* nuclear polyhedrosis virus genomic library which is able to *trans* activate promoters of reporter plasmids in transient expression assays. Using this assay, we found that three genes located between 83.7 and 7.5 map units of the *Autographa californica* nuclear polyhedrosis virus genome were involved in expression from the late capsid protein (*vp39*) and very late polyhedrin (*polh*) gene promoters. Two of these genes, *ie-1* and *ie-n*, *trans* regulate early genes in transient expression assays. Although *ie-1* was necessary and sufficient for expression from the early promoter in our assay, it was necessary but not sufficient for expression from the *vp39* and *polh* promoters. The presence of *ie-n* increased expression from the early, late, and very late classes of promoters tested. However, a third gene identified in this region was specifically required for expression from the *vp39* and *polh* promoters. This gene, a previously sequenced 630-nucleotide open reading frame, was renamed *lef-2* for late expression factor 2. We also found that other genes in the region between 83.7 and 7.5 map units were not required for expression from the promoters used in this assay, although we did not eliminate the possibility that they subtly modify expression. These genes include *pe-38* and *me53*, early genes with zinc finger-like motifs, and the upstream exon of *ie-0*, which specifies an alternate form of IE-1.

Three main transcriptional phases can be distinguished within the temporally regulated cascade of gene expression from the large, double-stranded DNA genomes of baculoviruses during infection of their host insect cells. The early phase precedes the onset of viral DNA replication and includes transcription of genes involved in viral DNA replication and regulation of early and late gene expression. The late and very late phases of transcription are dependent on DNA replication and include the expression of genes required for virus assembly and occlusion (for a review, see reference 33).

The mechanisms involved in the transitions from the early to late phase or from the late to very late phase are not clear. Early genes are transcribed by an alpha-amanitin-sensitive RNA polymerase (16, 23) present in uninfected cells (21); host RNA polymerase II is the most likely candidate for early gene transcription. Upon viral infection, early genes are transcribed in the presence of either cycloheximide or aphidicolin (8, 10, 22, 37, 40, 42), indicating that no newly synthesized viral products are required for early promoter recognition. Virus-specified factors, however, augment early gene expression (4, 13, 17, 19).

Late and very late genes, however, are transcribed by a virus-induced alpha-amanitin-resistant RNA polymerase activity (11, 16, 23). Late gene transcription is blocked by both cycloheximide and aphidicolin (10, 22, 37, 39, 45), reflecting a dependence on expression of early viral genes and DNA replication. The dependence of late gene expression on DNA replication is further supported by the observations that mutations in genes involved in DNA replication such as *p143*, a homolog of helicases (29), and *etl*, a homolog of proliferating cell nuclear antigen (*pcna*) (32), block or delay late gene expression (3, 8, 14). Viral genes specifically involved in the transcriptional or translational regulation of late and very late genes, however, have not been clearly identified.

The products of several *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) genes are thought to have gene-regulatory functions on the basis of their *trans*-regulatory activity in transient expression assays or the presence of structural motifs found in other known regulatory proteins. These genes are *ie-1*, *ie-0*, *ie-n*, *pe-38*, *cg30*, and *me53*. The product of *ie-1*, IE-1, is known to be a powerful *trans* activator of early gene expression in transient expression assays (1, 17, 19, 30, 38). The only known spliced gene in Ac*M*NPV, *ie-0*, has two exons (7, 26); the downstream exon is essentially *ie-1*, so the product of *ie-0*, IE-0, is an alternate form of IE-1 that contains an additional 54 residues at the amino terminus (7). In transient expression assays, *ie-0* or *ie-1* alone can *trans* regulate a number of early genes (1, 17, 19, 30, 38) and at least one late gene (20). This *trans* activation can be enhanced by *cis*-acting elements within short homologous regions (*hr*s) interspersed in the Ac*M*NPV genome (18). The *ie-n* gene product, IE-N, augments expression from some early promoters when IE-1 is limiting (4), while expression from *ie-n* is down-regulated by IE-1 (5). Although *ie-1* and *ie-n* were originally described as immediate early genes, transcription of early genes which IE-1 *trans* activates occurs in the presence of cycloheximide during virus infection, thus blurring the distinction between immediate early and delayed early transcriptional phases. An absolute requirement for newly synthesized IE-1 is observed only in transient expression assays with naked viral DNA; this requirement could be met by virus-borne proteins in a normal infection.

The other three putative regulatory genes, *pe-38* (27), *cg30* (40), and *me53* (25), are also transcribed early in infection. The putative amino acid sequences of both PE-38 and CG30 contain both zinc finger-like and leucine zipper motifs. The

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zinc finger-like motifs of PE-38 and CG30 are similar to those found in the herpes simplex virus promiscuous regulator, ICP0, and several other polypeptides having DNA-related functions (9). ME53 contains another type of zinc finger-like motif (25). However, a direct role in gene regulation has not been demonstrated for any of these gene products.

We have developed a transient expression assay to identify the viral genes required for late and/or very late gene expression. In this study, we determined which of the genes located between 83.7 and 7.5 map units (m.u.) on the physical map of the Ac*M*NPV genome are necessary for late and very late gene expression; this region includes the *ie-0* upstream exon and *ie-1*, *ie-n*, *pe-38*, and *me53* genes. The *ie-1* gene was found to be required for expression from the three promoters representing early, late, and very late transcriptional classes. The *ie-n* gene was found to augment expression from all three promoters. The *ie-0* upstream exon and *pe-38* and *me53* genes were not required in this assay for substantial expression from any of the promoters tested. A previously sequenced open reading frame (ORF) of 630 nucleotides (the 630 ORF) (35), however, was found to be required for transient expression from the late and very late promoters but not from the early promoter tested.

TABLE 1. Ac*M*NPV clone library used in mapping genes required for early, late, and very late gene expression

Clone[a]	Approx endpoint (m.u.)[b]	
	Left end	Right end
BC5	4.8–4.9	14.7–18.4
HL8	7.35–13.1	22.0–23.4
HL5	14.2–14.7	23.4–29.0
ETL7	20.1–22.0	33.5–34.8
PstH4	25.0–29.0	38.2–42.1
PstH1	32.9–34.8	42.8–45.4
PstH5	34.8–38.2	49.2–50.3
HC9	46.4–48.2	57.0–58.0
HC10	54.0–56.0	64.0–66.0
pXmaB	65.4	83.7
HK5	78.4–80.8	91.0–93.4
IE15	89.2–91.0	4.9–5.7

[a] All fragments except pXmaB were generated by partially digesting Ac*M*NPV with *Mbo*I, partially filling these sites, and ligating into a partially filled *Xho*I site of a lambda phage vector, LambdaGEM-11 (Promega). Plasmid pXmaB contains the *Xma*I-B fragment of Ac*M*NPV (see Materials and Methods).

[b] m.u. correspond to the physical map provided by O'Reilly et al. (33).

MATERIALS AND METHODS

Virus and cells. DNA from the wild-type L-1 strain (28) of Ac*M*NPV was utilized in this study. The virus was propagated either in *Trichoplusia ni* (cabbage looper) larvae or in the continuous *Spodoptera frugiperda* IPLB-SF-21 cell line (SF-21) (43). SF-21 cells were maintained at 27°C in TC-100 medium (GIBCO BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum and 0.26% tryptose broth (33).

Reporter plasmids. The three main reporter plasmids, pETCAThr5, pCAPCAT, and phcwt, all contain a portion of homologous region 5 (*hr5*) (36) upstream of an Ac*M*NPV promoter controlling expression of a reporter gene encoding chloramphenicol acetyltransferase (CAT). Viral polyadenylation signals are located downstream of the CAT gene in each plasmid. Reporter plasmids pCAPCAT (41) and phcwt (31) have been previously described and contain the late capsid protein gene (*vp39*) promoter and very late polyhedrin gene (*polh*) promoter, respectively. Reporter plasmid pETCAThr5 contains the *hr5* region from phcwt at the junction of pBluescript (Stratagene, La Jolla, Calif.) and Ac*M*NPV sequences upstream and downstream of the ETL ORF (8). The upstream region extends from the *Hin*dIII site at 31.0 m.u. to a *Sal*I linker which was inserted at position −5 with respect to the ETL translational start codon. In order to construct the reporter plasmid, the *Sal*I CAT gene cassette from pCM-1 (Pharmacia, Piscataway, N.J.) was inserted at this new *Sal*I site. Downstream of the CAT gene, there is an additional 1.8 kbp of Ac*M*NPV DNA starting at position +10 relative to the ETL translational start codon.

Two additional reporter plasmids, pCAP324VI$^+$X3CAT and phcwtdelH_3Nt, were utilized. pCAP324VI$^+$X3CAT contains the *vp39* promoter between −324 and −2 nucleotides with respect to the translational start codon (41) in place of the LSXIV promoter in vector pLSXIVVI$^+$X3 (44). The *Sal*I CAT gene cassette from pCM-1 was inserted at a *Sal*I site in pLSXIVVI$^+$X3 downstream of the *vp39* promoter. phcwtdelH_3Nt was constructed by subcloning the 2.5-kbp *Hin*dIII fragment of phcwt, extending from the *hr5* sequence to the Ac*M*NPV sequence at 4.1 m.u., in pBluescript. Thus, this plasmid is similar to phcwt but lacks the Ac*M*NPV region from 4.1 to 5.7 m.u.

Ac*M*NPV genomic plasmids. Plasmids pH_3G, pH_3F, pPstN, pXmaB, and pR1-I contain the Ac*M*NPV genomic fragments *Hin*dIII-G, *Hin*dIII-F, *Pst*I-N, *Sma*I-B, and *Eco*RI-I, respectively. pH_3G was cloned in pUC19 (46), pXmaB was cloned in pACYC177 (6), and pR1-I was cloned in pBR322 (2). pIE1/HC contains *ie-1* in a *Cla*I-*Hin*dIII fragment (94.7 to 96.9 m.u.) (19) cloned in pBR322. The remaining constructs utilized the plasmid vector pBluescript. Plasmid pPE-38 contains the Ac*M*NPV *Bgl*II-*Eco*RI fragment (98.4 to 100.0 m.u.), which includes *pe-38* (27). pBE42 has the 4.2-kbp *Eco*RI-*Bam*HI fragment between 0.0 and 3.3 m.u. subcloned from pR1-I, and pR1D1 has an *Eco*RI-*Mlu*I fragment (0.0 to 1.9 m.u.) subcloned from pBE42. p327, p630, and p603 contain the 327 ORF in an *Xho*I-*Sal*I fragment (1.5 to 2.2 m.u.), the 630 ORF in a *Mlu*I fragment (1.9 to 2.6 m.u.), and the 603 ORF in a *Sal*I-*Eco*RV fragment (2.4 to 3.0 m.u.), respectively (35). p630-del was constructed to disrupt the 630 ORF by deleting the 285-bp *Sal*I fragment within the 630 ORF from p630, blunt-ending with the DNA polymerase I large fragment (Klenow; Promega, Madison, Wis.), and religating. pBCNE contains the *lef-1* gene (34) within 1.4 kbp of Ac*M*NPV DNA between *Nru*I (7.5 m.u.) and *Eco*RI (8.65 m.u.).

DNA cotransfections and transient expression assays. SF-21 monolayers (2×10^6 cells per 60-mm dish) were cotransfected by the calcium phosphate coprecipitation method (33) with 2 μg of reporter plasmid, approximately 0.5 μg of Ac*M*NPV DNA or 0.5 μg of each DNA constituting the overlapping Ac*M*NPV library (Table 1), and 0.5 μg of each plasmid DNA added as specified for each cotransfection. Salmon sperm DNA was used to maintain the total concentration of DNA constant in each cotransfection mixture. After cotransfection, cells were maintained in complete TC-100 medium at 27°C for 24 h for cotransfections with pETCAThr5, 48 h for cotransfections with pCAPCAT or pCAP324VI$^+$X3CAT, or 72 h for cotransfections with phcwt or phcwtdelH_3Nt before being harvested. CAT assays were performed with 1/10 of the cell lysate as described elsewhere (15), with modifications (36).

RESULTS

Functional mapping of genes required for early, late, and very late gene expression. In order to identify genes which regulate the transitions between temporal phases of AcMNPV gene expression, we developed an assay which allowed the identification of specific genes required for expression from different temporal classes of AcMNPV promoters. This assay utilized reporter plasmids containing the CAT gene fused to an early, late, or very late AcMNPV promoter. Reporter plasmid pETCAThr5 containing the *etl* (8) promoter driving CAT was used to examine the requirements for early promoter activation. pCAPCAT (41) containing the promoter of the capsid protein gene, *vp39* (39), fused to CAT and phcwt (31) containing the promoter of the polyhedrin gene, *polh*, fused to CAT were used to examine the requirements for late and very late promoter activation, respectively. Both pCAPCAT and phcwt contained the promoter-CAT gene fusion within sequences flanking the AcMNPV *polh* gene from 2.4 to 5.7 m.u. None of these reporter plasmids exhibited significant CAT activity upon transfection into SF-21 cells in the absence of AcMNPV DNA (Fig. 1, lane 1).

When SF-21 cells were cotransfected with one of these CAT reporter plasmids and AcMNPV DNA, substantial levels of CAT activity were observed (Fig. 1, lane 2). A set of overlapping 10- to 15-kbp cloned fragments, which collectively represent the entire AcMNPV genome (Table 1), was able to substitute for intact AcMNPV DNA, and substantial levels of CAT activity were observed for the three temporal classes of promoters (Fig. 1, compare lanes 2 and 3). By removing specific clones from this library, the assay enabled us to assess the contribution of individual AcMNPV regions to gene expression from each temporal promoter class.

The *ie-1* and *ie-n* genes but not the *pe-38* gene are involved in late and very late gene expression. The IE15 clone (Table 1) contains several genes known or thought to be involved in promoter *trans* activation, including *ie-1*, *ie-n*, and *pe-38*, which are not found in the flanking HK5 or BC5 clone (Fig. 2). Upon cotransfection of pETCAThr5, pCAPCAT, or phcwt with the AcMNPV set of overlapping clones lacking the IE15 clone, no CAT activity was detected in transient expression assays with any of the temporal classes of promoters (Fig. 1, lane 4). The addition of pH_3G and pH_3F, which together span most of the IE15 clone (Fig. 2), reactivated expression from all three promoters tested (Fig. 1, lane 5).

The involvement of pH_3G, which contains both *ie-0* and *ie-1*, was tested by cotransfection of the reporter plasmids with pH_3G alone or with a subclone of pH_3G, pIE1/HC, containing only *ie-1*. The *ie-1* gene is known to be necessary and sufficient for expression from several other early promoters described as "delayed early" (17, 19). Cotransfection of pETCAThr5 with pH_3G alone allowed expression from this promoter as did cotransfection of pETCAThr5 with pIE1/HC alone (Fig. 1A, lanes 6 and 7, respectively). Thus, *ie-1* is necessary and sufficient for *etl* promoter activity. However, other genes were required to obtain expression from the late and very late promoters, since cotransfections with pH_3G or pIE1/HC alone (Fig. 1B and C, lanes 6 and 7, respectively) were not able to activate the *vp39* or *polh* promoter. Substitution of IE15 with pH_3G in the presence of the remaining AcMNPV library also did not result in reporter gene expression from pCAPCAT or phcwt (data not shown).

We then tested whether addition of *ie-1*, *ie-n*, and *pe-38* could substitute for IE15 in this assay and activate expression from the *vp39* and *polh* promoters. SF-21 cells were cotransfected with the AcMNPV library in which IE15 was replaced by pIE1/HC (containing *ie-1*), pPstN (containing *ie-n*), and pPE-38 (containing *pe-38*). Figures 1B and C (lane 8) show that these three plasmids were not able to replace IE15 in activating the *vp39* or *polh* promoter. These results indicated that there was an additional gene(s) within IE15 required for expression from the *vp39* and *polh* promoters.

High levels of CAT activity were obtained when pIE1/HC, pPstN, pPE-38, and pBE42 (0.0 to 3.3 m.u.) (Fig. 2) were simultaneously substituted for IE15 in the presence of the other AcMNPV library clones (Fig. 1B and C, lane 9). In order to determine whether each of these plasmids was necessary for expression from the late and very late promoters, we omitted one plasmid at a time. Cotransfection with the AcMNPV library lacking the IE15 clone but supplemented with pIE1/HC, pPstN, and pBE42 allowed expression from all three reporter plasmids (Fig. 1, lane 10). Therefore, the *pe-38* gene was not required for substantial *vp39* and *polh* promoter activities under these conditions. Omission of *ie-n* in the cotransfections reduced expression from all three promoters (Fig. 1, compare lanes 10 and 11). Thus, *ie-n* was not required for minimum expression from these promoters but augmented expression significantly. In the absence of *ie-1*, no expression from any of the promoters was observed (Fig. 1, lane 12), demonstrating that *ie-1* was essential for all gene expression in this assay. Finally, omission of pBE42 abolished *vp39* and *polh* promoter-driven gene expression but did not affect *etl* promoter-driven gene expression (Fig. 1, lane 13). Thus, pBE42 contains a gene(s) required for expression from these late and very late promoters but not from the early *etl* promoter.

The 630 ORF is required for late and very late gene expression. In order to delineate the gene(s) necessary for late and very late gene expression within the pBE42 clone, we obtained subclones from this plasmid and substituted them for pBE42 in cotransfections with the AcMNPV library lacking IE15 but containing pIE1/HC and pPstN. Figure 3 shows that pR1D1 (0 to 1.9 m.u.) (Fig. 2) was not able to substitute for pBE42 to activate expression from the late and very late promoters (Fig. 3, lane 6). The remaining AcMNPV sequences of pBE42 contain the 327-, 630-, and 603-nucleotide ORFs and a portion of the *polh* gene (35). Each ORF (except *polh*, which has been shown previously to be nonessential for late and very late gene expression) was tested individually (Fig. 3, lanes 7 to 9) and in pairs (data not shown). The 630 ORF but not the 327 or 603 ORF was able to substitute for pBE42 in activating the *vp39* and *polh* promoters (Fig. 3, lanes 7 to 9). In addition, the disruption of the 630 ORF by deleting a 285-bp region within the 630 ORF (p630-del) obliterated CAT activity from the late and very late promoters (Fig. 3, lane 10). Thus, the region between 93.4 and 2.6 m.u. contains three genes, *ie-1*, *ie-n*, and the 630 ORF (renamed *lef-2*), involved in *vp39* and *polh* promoter expression under these assay conditions.

Other ORFs between 83.7 and 7.5 m.u. are not required for late and very late gene expression. Reporter plasmids pCAPCAT and phcwt contain AcMNPV sequences from 2.4 to 3.3 m.u. and from 3.7 to 5.7 m.u. Thus, genes contained within these regions were supplied in the assay regardless of their omission with the IE15 clone. The genes contained in their entireties in these regions are the 603, 1629, and 588 ORFs (Fig. 2). The 1629 and 588 ORFs were tested independently by using reporter plasmids containing a *vp39* or *polh* promoter-CAT fusion with flanking AcMNPV sequences from

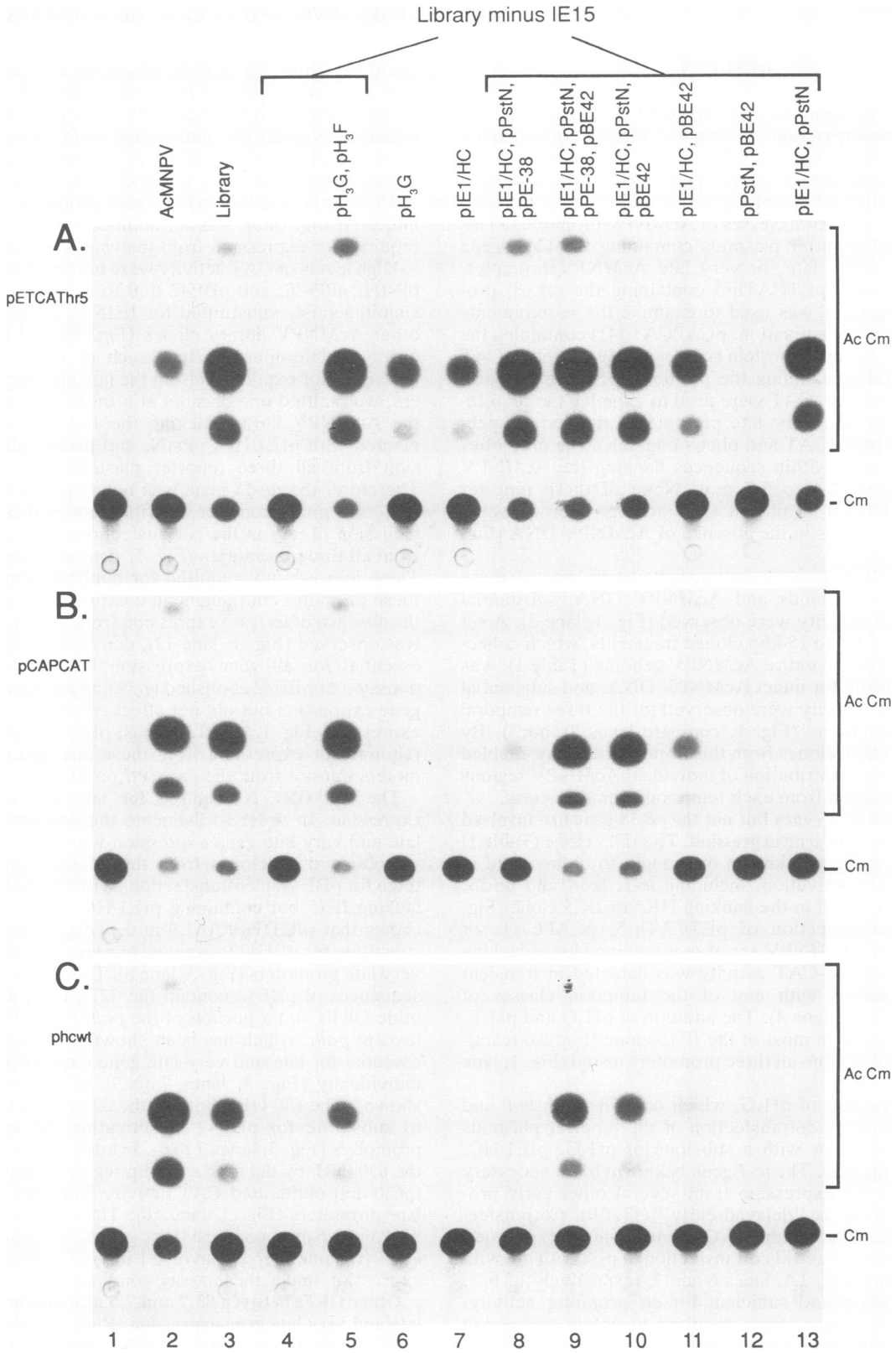

FIG. 1. CAT expression from early (A), late (B), and very late (C) Ac*M*NPV promoters in the presence or absence of genes from the IE15 region of Ac*M*NPV. SF-21 cells were transfected with pETCAThr5 (A), pCAPCAT (B), or phcwt (C), and cells were harvested at 24 h for pETCAThr5, 48 h for pCAPCAT, or 72 h for phcwt. Transfection mixtures contained the reporter plasmid only (lane 1), the reporter plasmid plus wild-type Ac*M*NPV DNA (lane 2), the entire overlapping Ac*M*NPV library (lane 3), or the Ac*M*NPV library lacking the IE15 clone with (lanes 5 and 8 to 13) or without (lane 4) additional genomic clones as specified above each lane. Transfection mixtures in lanes 6 and 7 contained only the reporter plasmid and the genomic clone specified above each lane. CAT activity in transfected cells was determined by enzyme assays; the acetylated products (Ac Cm) and unacetylated substrate (Cm) of each enzymatic reaction were separated by thin-layer chromatography and visualized by autoradiography.

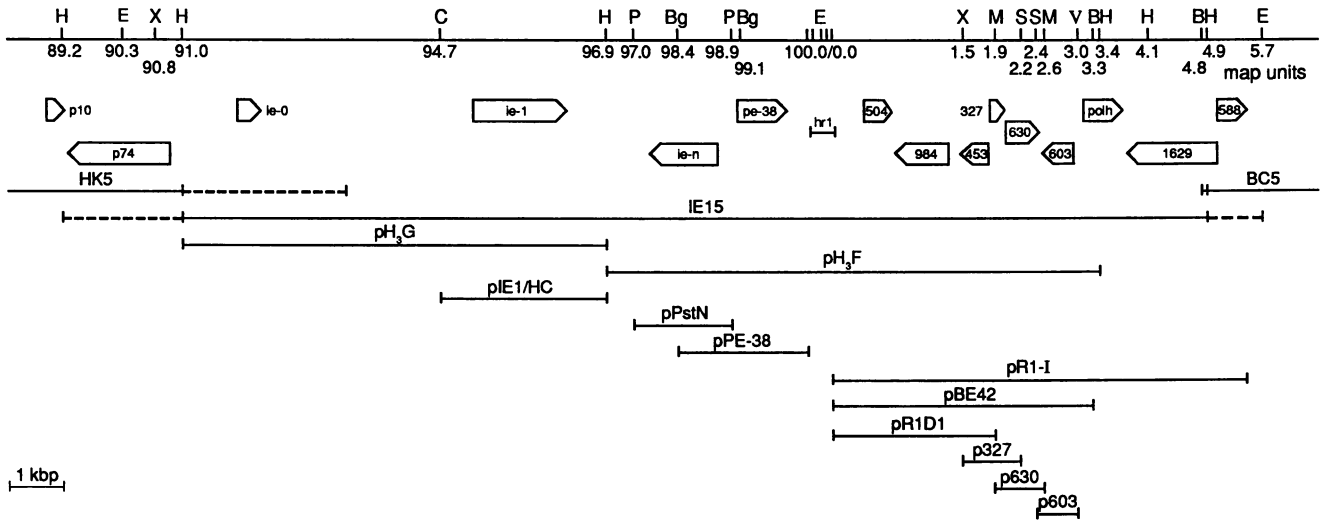

FIG. 2. Subclones in the region of the AcMNPV genome from 89.2 to 5.7 m.u. The names or sizes and orientations of relevant ORFs are shown below a physical map showing key restriction sites and respective m.u. Sizes of ORFs between 0 and 5.7 m.u. are given in base pairs and correspond to the sequence of Possee et al. (35). The entire IE15 lambda clone and portions of BC5 and HK5 lambda clones are shown by solid lines with broken lines at the ends indicating undefined endpoints of the clones. Plasmid subclones used in transient expression assays are shown below the library clones. H, *Hin*dIII; X, *Xho*I; C, *Cla*I; P, *Pst*I; Bg, *Bgl*II; E, *Eco*RI; M, *Mlu*I; S, *Sal*I; V, *Eco*RV; B, *Bam*HI. Not all sites in this region for *Cla*I, *Bgl*II, *Mlu*I, *Sal*I, and *Eco*RV are shown.

2.4 to 4.9 m.u. only (Fig. 4). These reporter plasmids do not contain the complete 1629 or 588 ORF but do contain the 603 ORF (and *polh* in the case of pCAP324VI$^+$X3CAT). These plasmids do not allow us to determine directly whether the 603 ORF contributes to gene expression in this assay, but this gene has been shown previously to be nonessential for viral infection and very late gene expression by mutational analysis (12).

To determine whether the 1629 ORF contributes to gene expression in this assay, each reporter plasmid was cotransfected with the overlapping library lacking IE15 but supplemented with pIE1/HC, pPstN, and pR1-I (0.0 to 5.7 m.u.) (pR1-I contains *lef-2* and the 1629 and 588 ORFs) (Fig. 4, lane 4) or pIE1/HC, pPstN, and p630 (Fig. 4, lane 5). Similar levels of expression were obtained with and without the 1629 ORF; thus, the 1629 ORF was not required for expression from the *vp39* or *polh* promoter.

The 588 ORF is contained in the BC5 library clone and may be contained in the IE15 library clone (Fig. 2), so the possible involvement of the 588 ORF had to be tested by omitting simultaneously both IE15 and BC5. However, we had observed that an additional gene within BC5 was required for late and very late gene expression in our assay (34). This gene, *lef-1*, was supplied in the plasmid pBCNE (7.5 to 8.65 m.u.). Four plasmids, pIE1/HC, pPstN, p630, and pBCNE, were sufficient to restore activity when both IE15 and BC5 were removed from the set of overlapping AcMNPV clones (Fig. 4, lane 8). Thus, the 588 ORF was not required for expression from the promoters tested.

Several additional genes are contained in the region overlapped by the IE15 and HK5 clones: the *ie-0* upstream exon and *me53*, an early gene between *ie-0* and *p74* (25). We tested for the requirement of this region in late and very late gene expression by cotransfecting pCAPCAT or phcwt with the library of clones lacking IE15 and HK5 but supplemented with plasmids which supply the three known necessary genes in IE15 (*ie-1*, *ie-n*, and *lef-2*) (Fig. 5, lane 6). When these three genes were supplied, CAT activity in the absence of IE15 and HK5 was comparable to that in the presence of the entire set of overlapping clones (Fig. 5, lane 2), indicating that neither *ie-0* nor *me53* was required for gene expression from these late or very late promoters. Because HK5 and IE15 can be replaced by *ie-1*, *ie-n*, and *lef-2*, only these three genes in the region from 83.7 m.u. to the *lef-1* gene in the BC5 clone (starting at 7.5 m.u.) are needed for expression from these two promoters in this assay.

DISCUSSION

We have developed a method for identifying AcMNPV genes required for late gene expression. This method is based on the ability of a set of overlapping clones, representing the entire AcMNPV DNA genome, to *trans* activate late and very late promoters in transient expression assays. Previous work had shown that in the presence of transfected intact AcMNPV DNA, the activity of the very late *polh* promoter of reporter plasmid phcwt is dependent on the presence of the promoter and responds to promoter mutations in a manner similar to that observed with viral infections (31, 36). Furthermore, transcription of the phcwt CAT gene in transient expression assays initiates at the correct site. Thus, this reporter plasmid as well as a similar plasmid, pCAPCAT, representative of the late transcriptional phase, was used to define genes involved in late and very late gene expression. The early promoter-based reporter plasmid served primarily as a control to distinguish genes involved specifically in the later phases of expression. By using these reporter plasmids, individual genes involved in *trans* activating expression were identified by the substitution of individual clones from the set of overlapping clones with smaller fragments to reconstitute *trans*-activating function.

In this study, we focused on the requirement for genes located within the 83.7- to 7.5-m.u. region. We found that three genes in this region, *ie-1*, *ie-n*, and *lef-2*, were involved in expression from the *vp39* and *polh* promoters. One of

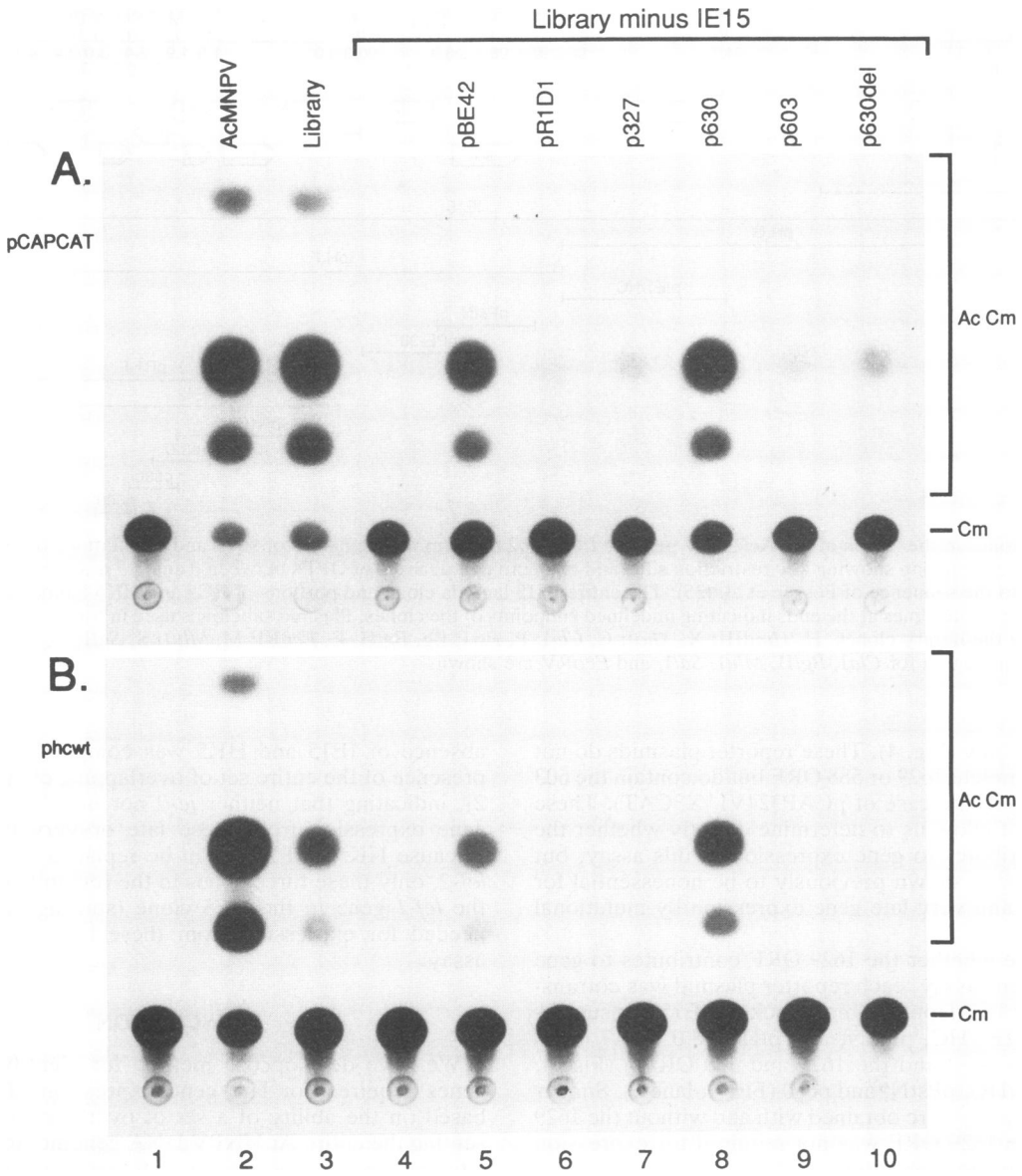

FIG. 3. Requirement of the 630 ORF for expression from the *vp39* and *polh* promoters. SF-21 cells were transfected with pCAPCAT (A) or phcwt (B) only (lane 1) or cotransfected with Ac*M*NPV DNA (lane 2), the entire Ac*M*NPV library (lane 3), or the Ac*M*NPV library lacking IE15 (lanes 4 to 10) but supplemented with plasmids pIE1/HC and pPstN (lanes 5 to 10). For lanes 5 to 10, the additional plasmid used is noted above each lane. CAT activity in transfected cells was determined by enzyme assays; the acetylated products (Ac Cm) and unacetylated substrate (Cm) of each enzymatic reaction were separated by thin-layer chromatography and visualized by autoradiography.

these genes, *ie-1*, was necessary but not sufficient for expression from late and very late promoters, although it was sufficient for expression from the early *etl* promoter. The latter observation is consistent with previous studies defining a role for *ie-1* in expression from other early promoters using transient expression assays (17, 19). Since *ie-1* is required for efficient expression of some early genes in transient expression assays (17, 19, 30), its requirement in activating late promoters may be an indirect one, involving the expression of other early *trans* regulators or gene products involved in DNA replication.

The second gene, *ie-n*, had also been observed previously to modulate early gene expression in transient expression assays. The reported effects of *ie-n* on early gene expression constitute an augmentation of expression in the presence of the *ie-1* gene product (4), and we also observed *ie-n* augmentation of early *etl* promoter activity in the presence of *ie-1*. The requirement for *ie-n* for expression from the *vp39* promoter was clear but not as striking as that for *ie-1*, since some expression from this promoter was obtained in the absence of IE-N. It is thus possible that the role of *ie-n* in the transition from early to late phase is to maximize expression of the early genes involved in late gene expression. Alternatively, *ie-n* may have a direct role in both early and late gene expression.

The third required gene, *lef-2*, has been identified previ-

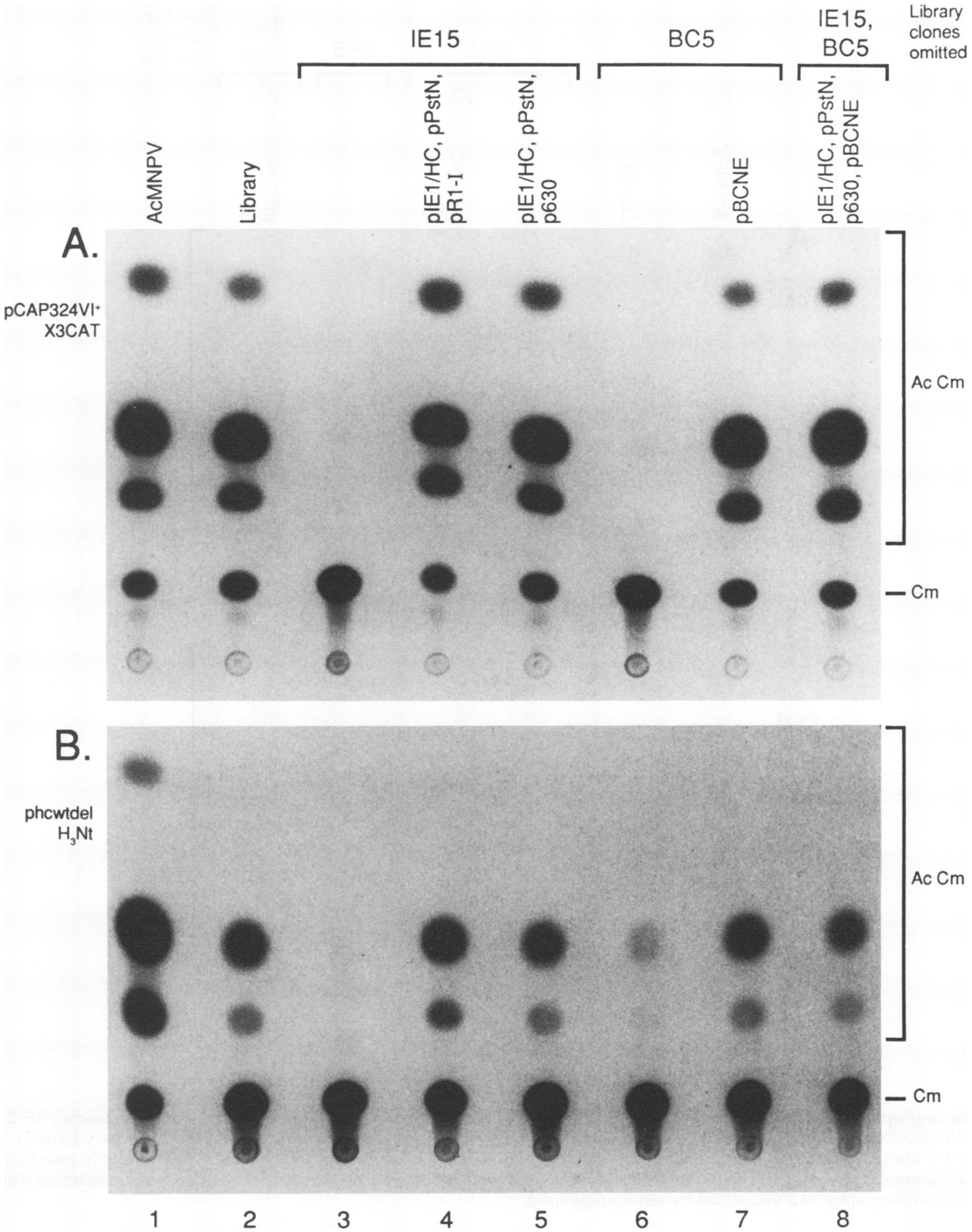

FIG. 4. A number of genes including the 1629 and 588 ORFs are not required for expression from the *vp39* and *polh* promoters. SF-21 cells were transfected with pCAP324VI$^+$X3CAT (A) or phcwtdelH_3Nt (B) in the presence of Ac*M*NPV DNA (lane 1) or the entire Ac*M*NPV library (lane 2). Omission of specific Ac*M*NPV library clones (IE15 and/or BC5) and addition of plasmids are noted above each lane. CAT activity in transfected cells was determined by enzyme assays; the acetylated products (Ac Cm) and unacetylated substrate (Cm) of each enzymatic reaction were separated by thin-layer chromatography and visualized by autoradiography.

ously only through DNA sequencing (35). Because *lef-2* appears to have no effect on *etl* promoter activity, *lef-2* is more likely than *ie-1* or *ie-n* to be directly involved in late gene expression, although it is possible that *lef-2* affects the expression of other untested early genes which in turn are required for late gene expression.

Homologs of *lef-2* have also been found near or adjacent to the *polh* genes of other nuclear polyhedrosis viruses (24, 47). The motif of cysteines and histidines in the sequenced portions of the LEF-2 homologs is conserved (47). However, LEF-2 shows no obvious homology to other known nonbaculovirus polypeptides and does not contain any known

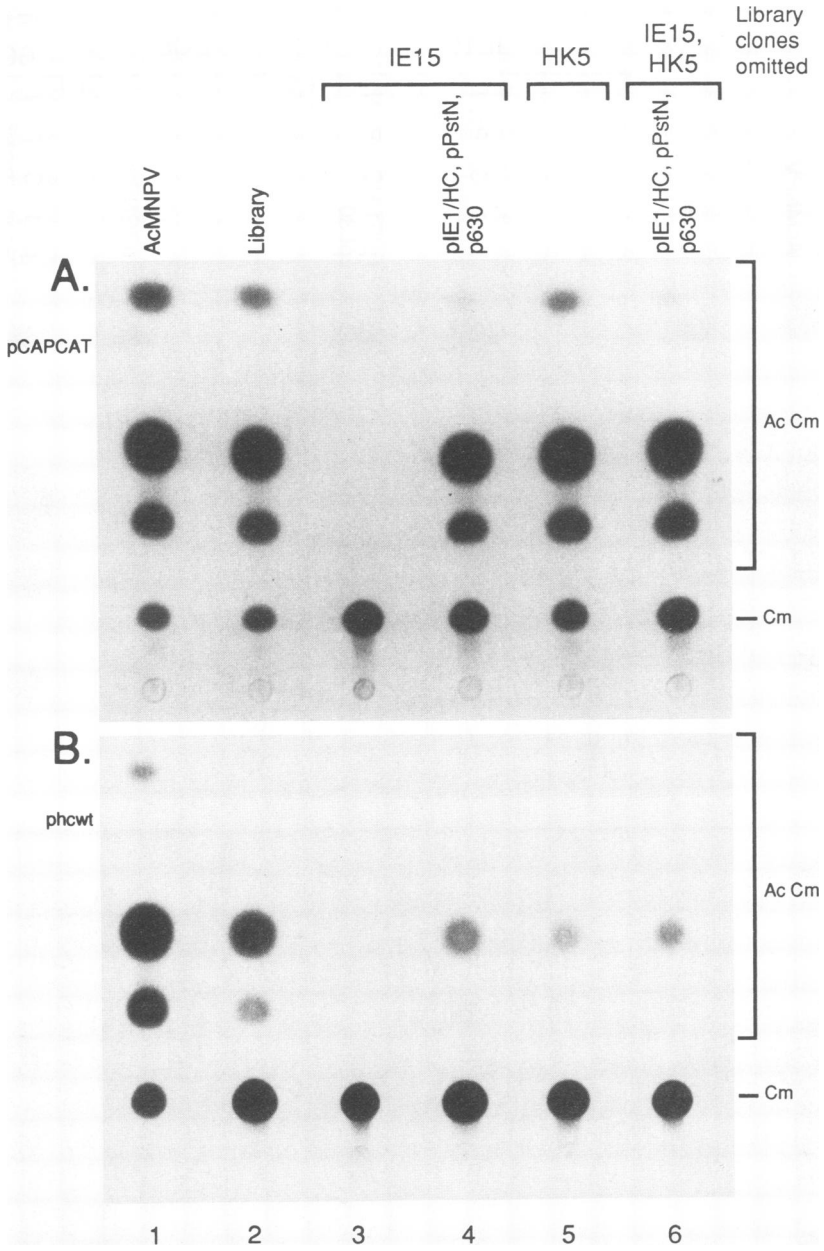

FIG. 5. The *ie-0* upstream exon and *me53* gene are not required for expression from the *vp39* and *polh* promoters. SF-21 cells were transfected with pCAPCAT (A) or phcwt (B) in the presence of Ac*M*NPV DNA (lane 1) or the entire Ac*M*NPV library (lane 2). Omission of specific Ac*M*NPV library clones (IE15 and/or HK5) and addition of plasmids are noted above each lane. CAT activity in transfected cells was determined by enzyme assays; the acetylated products (Ac Cm) and unacetylated substrate (Cm) of each enzymatic reaction were separated by thin-layer chromatography and visualized by autoradiography.

functional motifs. A transcriptional study of this general region of the Ac*M*NPV genome (29a) found that a 0.8-kb RNA is transcribed by 2 h postinfection and a 1.4-kb RNA is transcribed by 6 h postinfection. Numerous transcripts are observed at very late times in this region, but since none of these transcripts have been mapped, the relevance of these observations to *lef-2* transcription remains obscure. The first putative translational initiation codon of the *lef-2* ORF overlaps with the upstream 327 ORF (35; also see appendix I of reference 33). Two TAAG sequences are located within the 327 ORF, and an additional TAAG sequence is located 42 nucleotides upstream of the putative 327 ORF translational start site. TAAG sequences are the consensus start site for late and very late transcription. Thus, it is possible that both of these ORFs are transcribed late in infection although they may also have early promoters as well.

In the process of determining that three genes between 83.7 and 7.5 m.u. were necessary for expression from the *vp39* and *polh* promoters, we also found that a number of genes in this region were not required for significant transient expression from these promoters. PE-38 (27), which contains a zinc finger-like motif and a leucine zipper, was

shown not to be required for the activities of these promoters; it may, however, play a more subtle modulating role. For example, this assay may not easily distinguish genes involved in turning off gene expression since CAT is a relatively stable protein. Alternatively, *pe-38* may play a role in the regulation of other AcMNPV promoters which have not yet been tested.

The 1629 ORF, which appears to be essential for virus replication (35), was also not required for *etl*, *vp39*, or *polh* promoter-driven expression in these transient expression assays. The 504 ORF and the 588 ORF were also not required in this transient expression assay. Finally, neither the *ie-0* upstream exon nor the *me53* gene (25) was required for *vp39* or *polh* promoter-driven expression in this assay.

We utilized the *etl*, *vp39*, and *polh* promoters as examples of early, late, and very late promoters, respectively, to find factors between 83.7 and 7.5 m.u. that are required for expression from each temporal class of promoter. The regulatory cascade controlling the transcriptional program depends on the expression of factors from an earlier phase(s) for expression of a subsequent phase. Our results suggest that factors acting only on the very late expression phase may not be present in this region. Since expression from the *polh* promoter is not as high as expression from the *vp39* promoter in our assay, we are not yet certain that this assay will detect genes specifically required for the very late activation of the *polh* promoter. At the present time, we have tested only one promoter which we thought would be representative of each of the three main transcriptional classes; it will be of interest to determine whether these requirements can be extrapolated to other promoters within the same class.

Since late and very late gene expression is dependent on viral DNA replication in vivo, the genes required for late and very late gene expression in this transient expression assay may also include those involved in viral DNA replication. It is not yet known, however, whether prior DNA replication is required for baculovirus late gene expression in transient expression assays. The *hr5* sequence, which has *cis*-acting effects on both transcription (18) and DNA replication (34a), was included in all our reporter plasmids, but we do not yet know the full extent of its effects on our assay. Preliminary studies indicate that the presence of the *hr5* sequence in *cis* has a marked stimulatory effect on expression from the early *etl* promoter. Prior studies (36) with pcwt, a plasmid identical to phcwt but lacking the *hr5* sequence, showed that the presence of the *hr5* sequence has very little effect (less than twofold) on expression from the *polh* promoter in transient expression assays. Because the AcMNPV homologous regions, particularly *hr5*, have been found to serve as origins of baculovirus DNA replication (34a), this result suggests, but does not prove, that DNA replication and plasmid copy number are exerting little effect on expression in this system. (It is possible that other sequences in phcwt, including plasmid vector sequences, might be providing a replication origin.)

We expect that our assay should allow us to locate the genes which encode part or all of the alpha-amanitin-resistant RNA polymerase involved in late gene transcription and key accessory genes involved in late gene transcription or translation. The products of the genes identified with this assay will be best characterized in a baculovirus in vitro transcription system.

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