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Functional Mapping of *Autographa californica* Nuclear Polyhedrosis Virus Genes Required for Late Gene Expression

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A plasmid containing the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of an *Autographa californica* nuclear polyhedrosis virus (AcNPV) late gene promoter was constructed. This plasmid (pL2cat) also contained the AcNPV *hr5* enhancer element. Transient-expression assay experiments indicated that the late promoter was active in *Spodoptera frugiperda* cells cotransfected with pL2cat and AcNPV DNA but not when pL2cat was transfected alone. Low levels of CAT activity were observed in cells cotransfected with pL2cat and pIE-1 DNAs. However, CAT activity was not induced in a similar plasmid which lacked the *cis*-linked enhancer element, indicating that the enhancer was required for expression of the late gene. Cotransfection mapping of p*Pst*I clones of AcNPV DNA indicated that the p*Pst*I-G clone of viral DNA contained a factor which further stimulated late gene expression 3- to 10-fold. Transient-expression assay analysis of subclones of p*Pst*I-G localized the *trans*-active factor to a 3.0-kilobase *Xba*I fragment. The nucleotide sequence of this fragment was determined and found to contain three potential open reading frames. A computer-assisted search of a protein database revealed no closely related proteins. One of the predicted amino acid sequences contained potential metal-binding domains similar to those found in nucleic acid-binding proteins. Subcloning and subsequent CAT assays indicated that two of the open reading frames were required for the activation of pL2cat. Nuclease S1 mapping of infected and transfected RNAs indicated that the two open reading frames were transcribed as delayed-early genes. Quantitative nuclease S1 analysis and differential DNA digestion of recovered plasmids indicated that the activation of pL2cat was not due to an increase in steady-state levels of mRNA replication of the viral DNA.

The expression of *Autographa californica* nuclear polyhedrosis virus (AcNPV) genes in infected cells is coordinately regulated in a cascade fashion. The immediate-early (α) genes are expressed in the absence of de novo viral protein synthesis (15). Functional α gene products, particularly the IE-1 gene product, are required for the expression of delayed-early (β) genes (10, 12). *trans*-Activation of the β gene 39K by IE-1 is enhanced by the presence of an AcNPV repeated element *cis* linked to the 39K promoter (11). IE-1 is expressed very early in the virus life cycle (12). Maximal transcription is detected by the end of the adsorption period, and steady-state levels of message are maintained for at least 24 h. As yet, it is unknown whether transcription of IE-1 or translation of IE-1 or both continues throughout infection. By 12 h postinfection, transcripts which overlap part or all of the IE-1 gene are expressed at levels equivalent to IE-1. Since the late (γ) RNAs are complementary to IE-1 mRNA, they possess the ability to hybridize and thereby inhibit the translation of IE-1. Therefore, the temporal regulation of this late promoter and the role of IE-1 in that regulation are of great interest.

Little is known concerning the factors which regulate late gene expression in AcNPV-infected cells. Expression of the late genes is normally linked to DNA replication, although it is not clear whether γ genes can be transcribed in the absence of DNA replication. In cells treated with cytosine arabinoside, AcNPV structural genes were synthesized and viral capsids were formed, although at a reduced rate when compared with that in untreated, uninfected cells (6). However, in another set of experiments which used aphidicolin to inhibit DNA replication, transcription of γ genes was not detected (18). Another factor which may control temporal expression is a virus-induced or virus-modified RNA polymerase (7). AcNPV early gene expression is sensitive to α-amanitin, indicating that α genes are transcribed by the host RNA polymerase II. During the late phase, viral transcription gradually shifts to α-amanitin resistance. Transcription of viral genes during the occlusion phase (very late or δ genes) is completely resistant to α-amanitin, although it is not clear whether γ gene transcription is sensitive or resistant to α-amanitin.

In the large DNA viruses, regulation of late gene expression involves both *trans*-acting early proteins and *cis*-acting DNA sequences in the promoter region of late genes. Regulation of the major late promoter of simian virus 40 is perhaps best understood. In a normal infection, simian virus 40 T antigen increases late gene expression directly by binding to viral DNA and indirectly through genome amplification (2, 14). The stimulation of late gene expression by T antigen is independent of and can occur in the absence of DNA replication. In contrast, adenovirus late genes are not expressed in the absence of viral DNA synthesis. Superinfection experiments indicate that parental DNA cannot serve as a template for late gene expression (23). The factors which restrict adenovirus late gene expression from parental DNA are not well understood but may involve the structure of the DNA or the components with which it is associated. In herpesvirus, the late genes form two subclasses, γ1 and γ2, which differ in their dependence on viral DNA synthesis for their expression (19). Inhibition of viral DNA replication causes only a moderate reduction in the accumulation of γ1 mRNAs, whereas γ2 mRNAs are not detected in the absence

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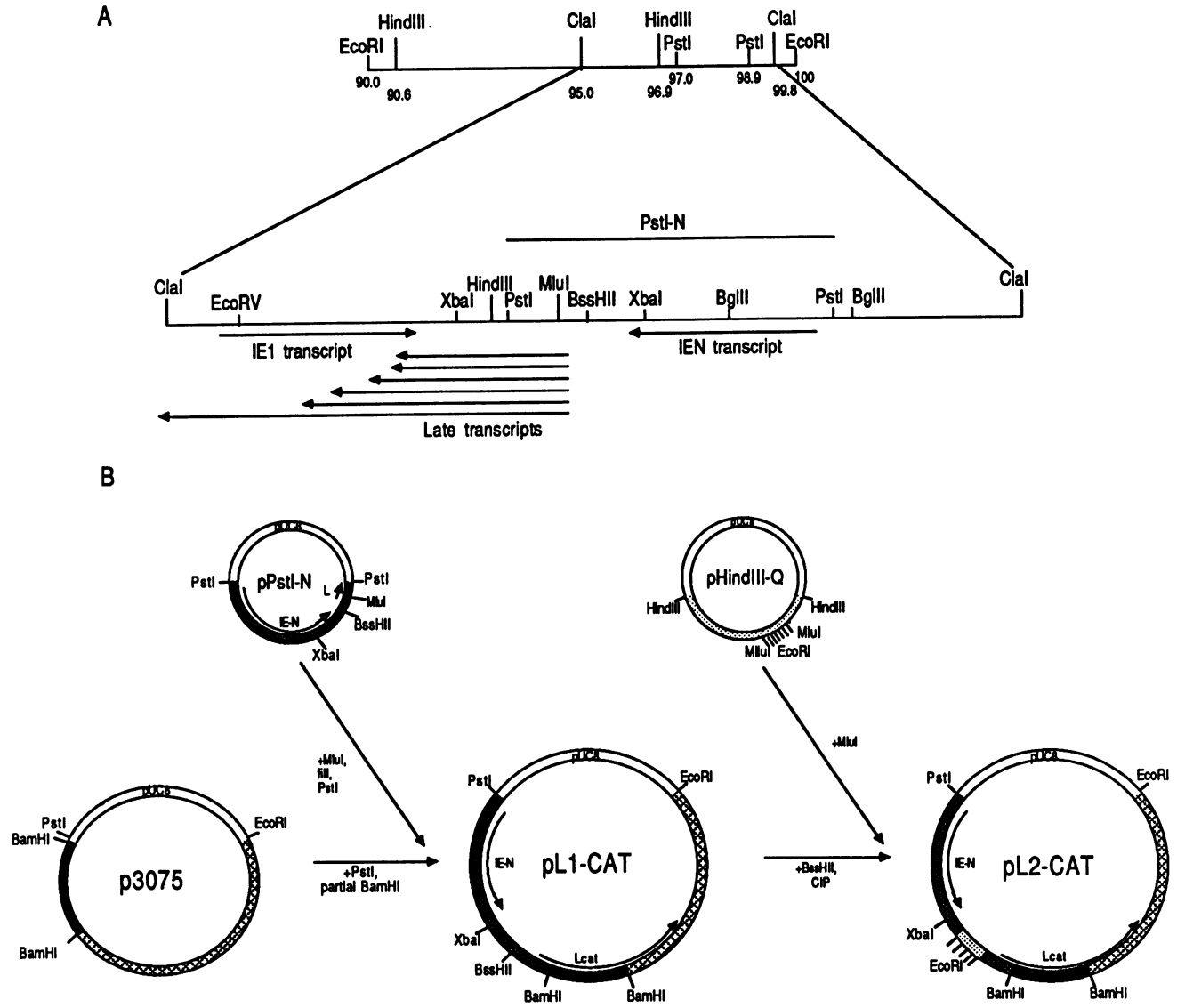

FIG. 1. Restriction map of *Eco*RI-B (A) and construction of chimeric plasmids (B). (A) A restriction map of EcoRI-B is shown with an enlarged map of the *Cla*I subfragment. Map locations of relevant sites in *Eco*RI-B are indicated in map units below the line. The transcripts mentioned in the text are indicated, with the direction of transcription denoted by arrows. (B) pL1cat was constructed by cloning the 2,350-bp *Pst*I-*Mlu*I fragment of p*Pst*I-N (shaded bars) upstream of the CAT gene (black bars) in p3075. The resultant plasmid contains the CAT gene 89 bp downstream of the transcriptional start site for the late promoter. The transcription termination and polyadenylation signals are derived from polyhedrin sequences (cross-hatched bars). pL2cat was constructed by cloning the 484-bp *Mlu*I fragment of p*Hin*dIII-Q containing *hr5* into the unique *Bss*HII site of pL1cat.

of viral DNA synthesis. The synthesis of γ1 and γ2 RNAs is *trans*-activated by the α genes ICP4 and ICP27, which also regulate β gene transcription (4, 20). Transcriptional regulation of the γ2 genes may be both negatively regulated early in infection by the β gene ICP8 and positively regulated late in infection by additional factors (8).

Here we report that the expression of the IE-1 antisense late promoter was activated at a low level by IE-1 in the presence of an AcNPV enhancer *cis* linked to the late gene. Expression of the late gene was further stimulated by *trans*-active factors which map to the *Pst*I G fragment of AcNPV DNA. The increase in expression of the late gene was not reflected by an increase in steady-state levels of late message and occurred in the absence of DNA replication.

TABLE 1. Expression of the plasmids used in this study[a]

Transfected plasmid or DNA	CAT activity (pmol/min per 10^6 cells)
pL1cat	0.0
pL1cat + AcNPV DNA	131.6
pL1cat + pIE-1	0.0
pL2cat	0.0
pL2cat + AcNPV DNA	626.4
pL2cat + pIE-1	2.2

[a] *S. frugiperda* cells were transfected with 1 μg of each plasmid or DNA, and CAT assays were performed 48 h posttransfection. For accurate quantitation of CAT levels, cell extracts were diluted so that 30% or less of the input chloramphenicol was acetylated.

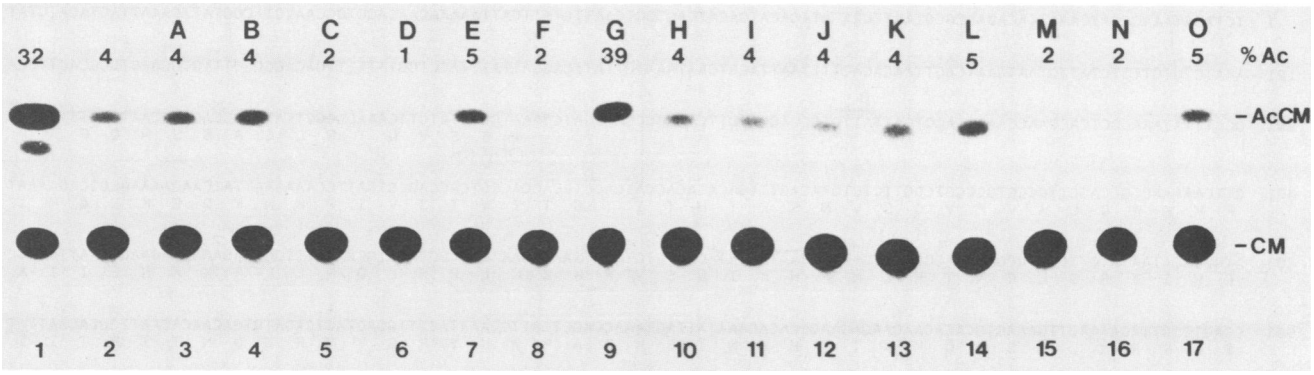

FIG. 2. Functional mapping of a *trans*-active factor which stimulates late gene expression. *S. frugiperda* cells were cotransfected with 1 μg of pL2cat and 1 μg of viral DNA (lane 1), pIE-1 (lane 2), or pIE-1 and the indicated p*Pst*I clones (lanes 3 to 17). After 48 h, cell extracts were prepared and CAT activity was determined by quantitation of the acetylated (AcCM) and unacetylated (CM) forms of chloramphenicol. The percent acetylation (%Ac) is indicated above each lane. The lysate used in lane 1 was diluted 10-fold prior to analysis.

MATERIALS AND METHODS

Transient-expression assay. The conditions for cell culturing and transfections were described previously (10). Chloramphenicol acetyltransferase (CAT) assays were performed as previously described except that the cells were harvested 48 h posttransfection (9, 10). Total cell RNA was purified and nuclease S1 analysis was performed as previously described (3, 10, 25).

Construction of recombinant plasmids. The plasmids containing the CAT gene under the control of an AcNPV late gene (Fig. 1) were constructed by standard procedures (16). An abundant late transcript which partially overlaps the immediate-early gene IE-1 has been described previously (12). The location of this transcript relative to IE-1 and another immediate-early gene, IE-N (D. D. Carson, L. A. Guarino, and M. D. Summers, Virology, in press), is shown in Fig. 1a. The *Pst*I N fragment (97.0 to 98.9 map units) of AcNPV DNA contains the promoter and 5′ end of the late gene as well as the entire IE-N gene. The complete sequence of this plasmid will be presented in a subsequent manuscript (D. D. Carson et al., manuscript in preparation). Plasmid p*Pst*I-N, containing the *Pst*I N fragment of AcNPV DNA cloned into the *Pst*I site of pUC8, was digested with *Mlu*I and then treated with the Klenow enzyme in the presence of deoxynucleotides to repair the ends. The digested plasmid was then incubated with *Pst*I. The resulting 2.4-kilobase (kb) fragment with one *Pst*I end and one blunt end was cloned into plasmid p3075 which had been completely digested with *Pst*I and partially digested with *Bam*HI. The resulting construct, pL1cat, contains the CAT gene under the control of the late promoter and the 3′ transcriptional signals for polyhedrin downstream of the CAT gene. To construct pL2cat, we cloned the 484-base-pair (bp) *Mlu*I fragment of p*Hin*dIII-Q containing the *hr5* enhancer region (11) into the *Bss*HII site upstream of the late promoter.

DNA sequence analysis. Both *Xba*I-*Bam*HI fragments of p*Pst*I G were subcloned into M13mp18 and M13mp19 (26). Progressive deletions were generated by the exonuclease III procedure (26) and sequenced by the dideoxy chain termination procedure (21). The 212-bp *Nsi*I fragment of p*Pst*I G which overlaps the *Bam*HI site was cloned into the *Pst*I site of M13mp18 in both orientations and sequenced. The sequences were compiled and analyzed by the programs of Devereaux et al. (5). The entire sequence was determined on both strands, and each base was sequenced an average of 4.5 times.

Analysis of DNA replication in transfected cells. *Spodoptera frugiperda* cells (10^6) were transfected with 1 μg of plasmid or viral DNA. After 48 h, the DNA was extracted by the procedure of Hirt (13). The extracted DNA was purified by phenol extraction and then ethanol precipitated. The precipitated DNA was suspended in 60 μl of TE (10 mM Tris [pH 7.5], 1 mM EDTA). Aliquots (10 μl) of plasmid DNA were digested with *Xba*I and *Mbo*I or *Dpn*I and separated on 1% agarose gels. The DNA was transferred to nitrocellulose (22) and probed with pUC8 DNA uniformly labeled with [α-^{32}P]dATP by nick translation by standard procedures (16).

RESULTS

Enhancer requirement for late gene expression. To determine whether one of the AcNPV enhancer elements located

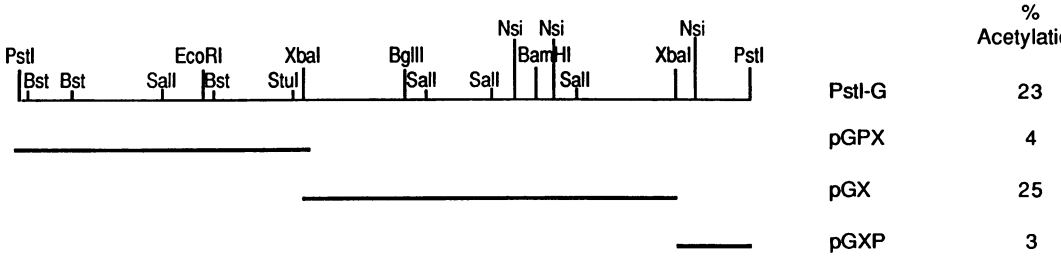

FIG. 3. Functional mapping of subclones of p*Pst*I-G. The indicated *Xba*I subclones of p*Pst*I-G were tested for *trans*-activation of pL2cat as described in the legend to Fig. 2. pGX = pPGX.

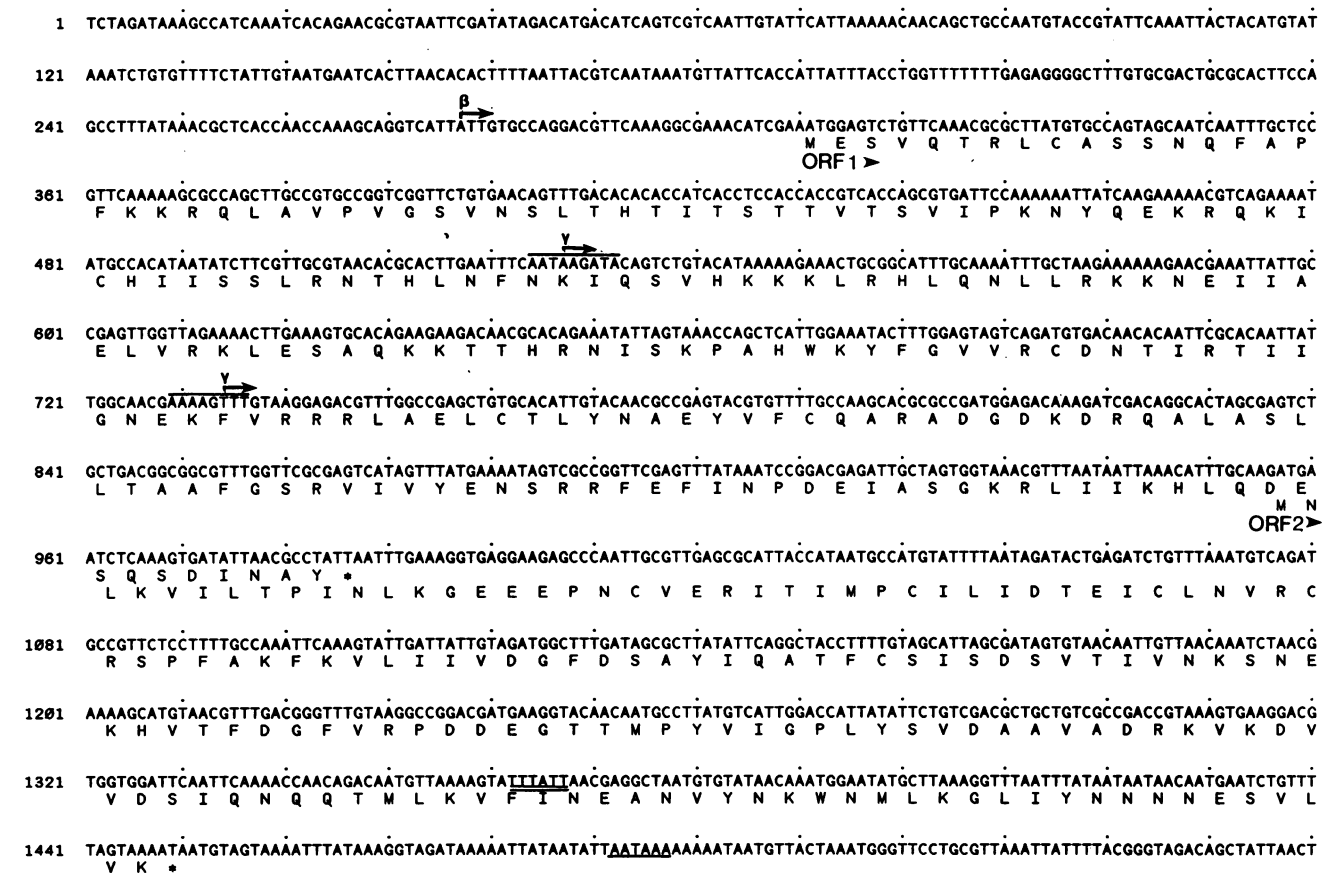

FIG. 4. Nucleotide sequence of the *Xba*I subclone of p*Pst*I-G and the predicted amino acid sequences of three major ORFs. The single-letter amino acid code is used. The locations of 5′ ends are indicated and designated β for delayed early and γ for late. Putative polyadenylation signals in the top strand are underlined; polyadenylation signals in the opposite strand are double underlined.

in the homologous regions was required for late gene expression, we cotransfected *S. frugiperda* cells with AcNPV DNA and plasmid pL1cat or pL2cat. CAT activity was fivefold higher in the cells transfected with pL2cat, containing the *hr5* enhancer, than in the cells transfected with pL1cat, lacking the enhancer (Table 1). Neither plasmid was active when transfected in the absence of viral DNA. A low level of CAT was induced when pL2cat was cotransfected with pIE-1, while pL1cat had no activity when cotransfected with pIE-1. The level of activity seen in pL2cat- and pIE-1-cotransfected cells was approximately 0.01% the level observed in pL2cat- and AcNPV-cotransfected cells, indicating that pIE-1 alone was not sufficient for maximal induction of late gene expression.

***trans*-Activation of pL2cat.** To functionally map the AcNPV gene(s) which stimulates late gene expression, we cotransfected pL2cat with pIE-1 and individual cloned *Pst*I fragments of AcNPV DNA. Cotransfection of pIE-1, pL2cat, and p*Pst*I-G induced 10-fold-higher levels of CAT expression than did cotransfection of pIE-1 and pL2cat alone (Fig. 2). Some p*Pst*I clones consistently inhibited CAT activity relative to the levels seen with pIE-1 alone. This observation was not further examined.

To define the region of p*Pst*I-G which contained the activating factor, we tested three *Xba*I subclones of p*Pst*I-G for their ability to *trans*-activate pL2cat in the presence of pIE-1 (Fig. 3). The results indicated that the *trans*-active region was the middle 3.7-kb *Xba*I region, hereafter referred to as pPGX.

DNA sequence analysis of the *trans*-active fragment. To learn more about the nature of the *trans*-active factor, we determined the nucleotide sequence of the *Xba*I fragment. The complete nucleotide sequence yielded 3,722 nucleotides. The predicted amino acid sequences of three long open reading frames (ORFs) are shown in Fig. 4. ORF-1 and ORF-2 read from left to right on the standard genome map. Ten amino acids at the C terminus of ORF-1 overlap the N terminus of ORF-2. ORF-3 reads from right to left on the standard map and is separated from ORF-2 by 166 nucleotides (nt).

ORF-1 encodes a highly basic protein (charge +20) that contains 226 amino acids and has a predicted molecular weight of 25,891. ORF-2 encodes the smallest of the three proteins, which contains only 164 amino acids, has a predicted molecular weight of 18,464, and is slightly acidic. ORF-3 encodes a protein that has a molecular weight of 40,865, is composed of 353 amino acids, and has a net neutral charge.

A computer-assisted search of the National Biomedical Research Foundation protein sequence database, release 10, detected no strong homology of the three ORFs with any of more than 4,000 published amino acid sequences. However, ORF-3 contained two pairs of short amino acid sequences that were bracketed by cysteine and histidine in the manner

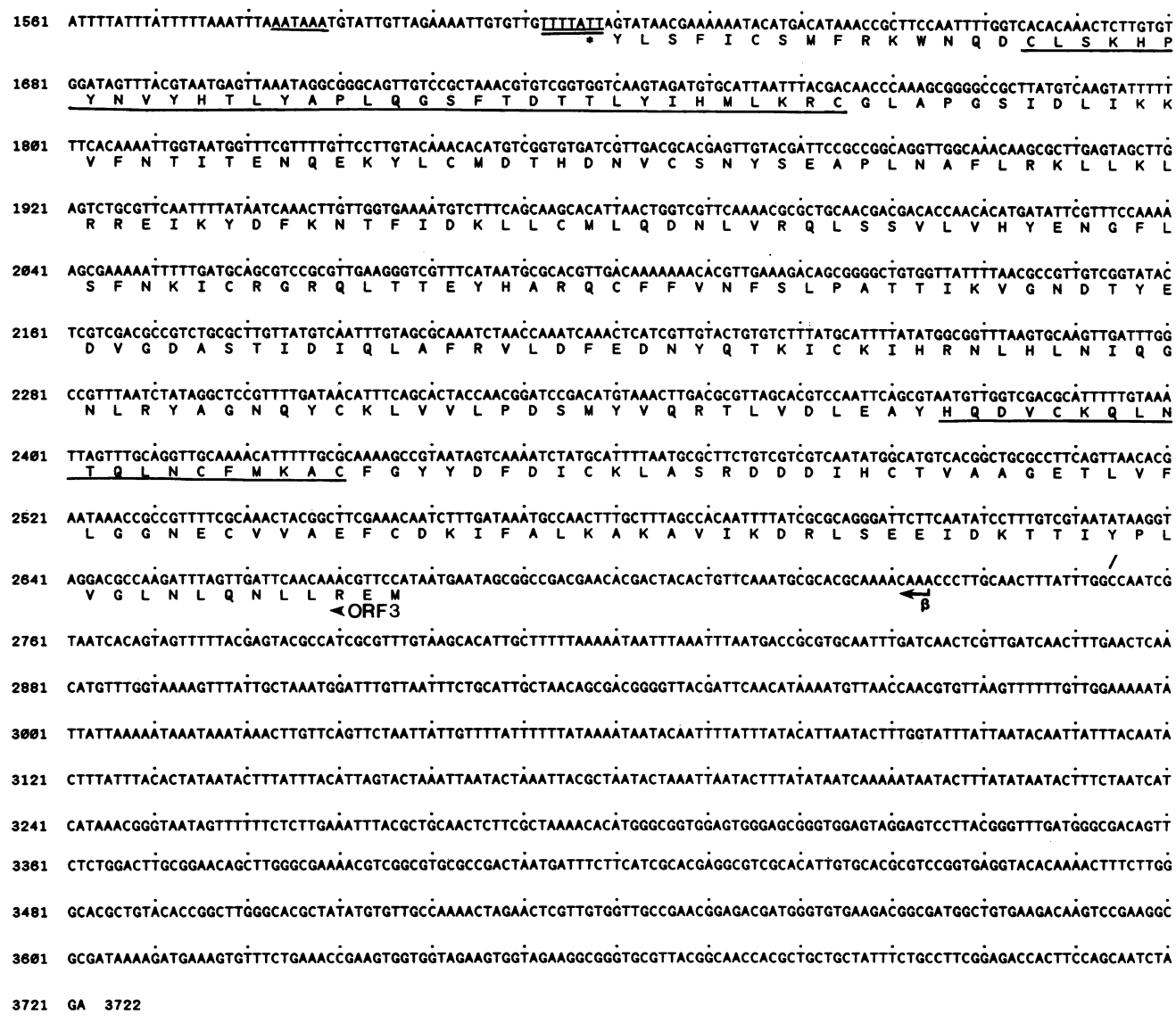

FIG. 4—*Continued*

of metal-binding domains found in nucleic acid-binding proteins (1).

Transcriptional activity of p*Pst*I-G. To determine which of the ORFs was transcribed early in infection, we performed quantitative nuclease S1 mapping with 6- and 18-h RNAs and with RNA purified from cells transfected with p*Pst*I-G alone and with p*Pst*I-G and pIE-1 (Fig. 5). To map the 5′ ends of the rightward transcriptional unit, we hybridized a 1,058-bp *Bgl*II-*Xba*I fragment, exclusively 5′ end labeled at the *Bgl*II site, with total cell RNA, treated the hybrid with nuclease S1, and analyzed it on 7 M urea–4% polyacrylamide gels. One major band of approximately 790 nt was detected with 6-h RNA and with RNA transfected with p*Pst*I-G and pIE-1. No protected fragments were seen with uninfected-cell RNA or with RNA transfected with p*Pst*I-G alone, indicating that these transcripts should be classified as delayed early. A more accurate determination of the map location of the 5′ ends of these transcripts was obtained with exonuclease III-generated M13 DNAs (data not shown). The nuclease S1-protected bands were analyzed on sequencing gels next to a sequence ladder of the M13 probe. The data indicated that the β transcript initiated 787 bp upstream of the *Bgl*II site or 34 bases upstream of the initiating AUG codon for ORF-1.

Two additional bands of 538 and 330 nt were observed after hybridization with 18-h RNA. These late transcripts initiated at sequences similar to the consensus sequence, AATAAGTATT, located near the 5′ ends of highly expressed late genes (17). The start sites for the late transcripts were found to be AATAAGATA and AAAAGTTT, respectively.

The heterogeneous 3′ ends of the rightward transcriptional unit were mapped with a 2,657-bp *Bgl*II-*Xba*I fragment specifically labeled at the *Bgl*II site. Three fragments were resistant to nuclease S1 treatment. The major 542-nt band indicated that most transcripts terminate 150 bp downstream of the termination of ORF-2, which is 18 bp downstream of the conserved polyadenylation signal, AATAAA. The 449-nt band corresponds to transcript termination 19 bp downstream of a polyadenylation signal and 59 bp downstream of

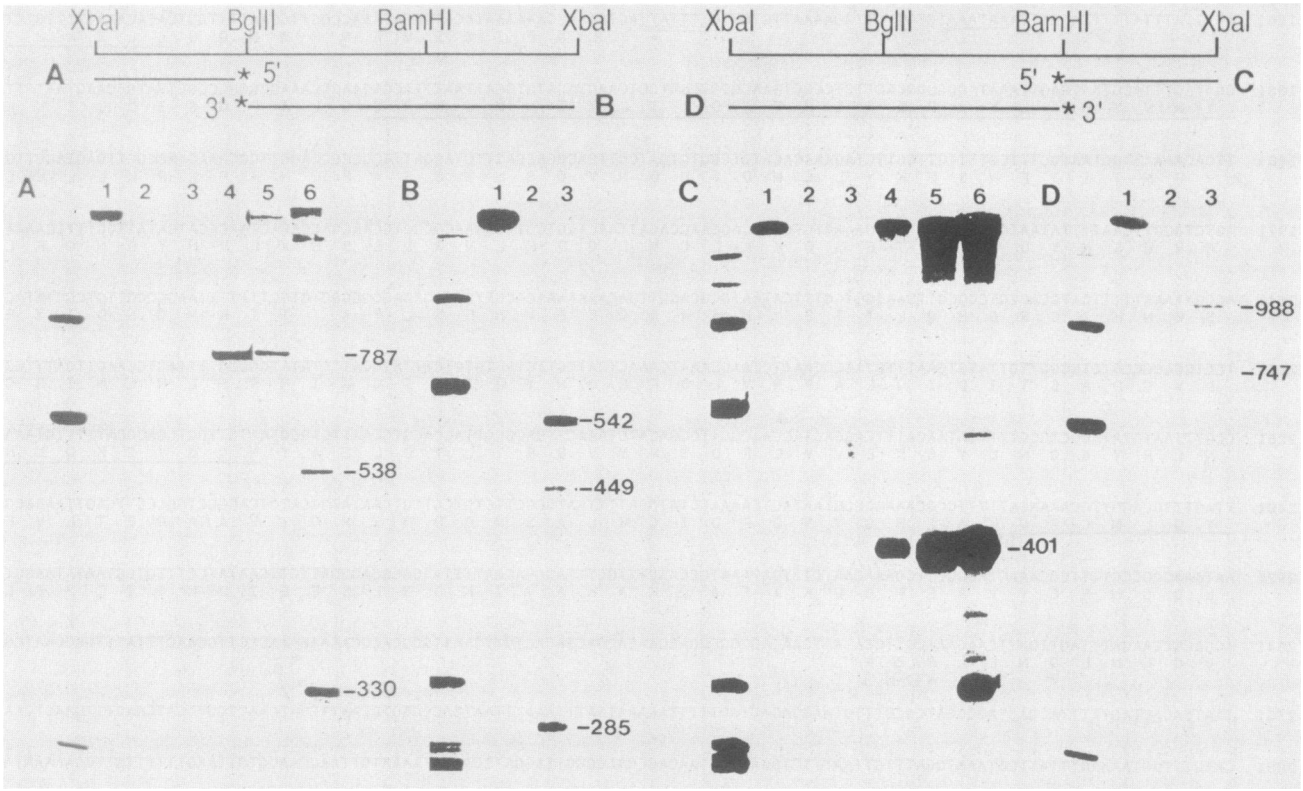

FIG. 5. Nuclease S1 mapping of the 5′ and 3′ ends of the transcripts which map to the *Xba*I fragment of p*Pst*I-G. After treatment with nuclease S1, the resistant fragments were denatured and analyzed on a 4% polyacrylamide–7 M urea gel. The molecular markers were *Hae*III fragments of ϕX174 DNA (first lane in each panel). The sizes of nuclease S1-resistant fragments are indicated on the right. The diagrams at the top indicate the map locations of the probes and the positions of the end labels. (A) The 5′-end-labeled probe (lane 1) was a 1,059-bp *Xba*I-*Bgl*II fragment, exclusively labeled at the *Bgl*II site, hybridized with 10 μg of total cell RNA isolated from uninfected *S. frugiperda* cells (lanes 2 and 8), with 1 μg of poly(A)$^+$ RNA from cells transfected with p*Pst*I-G (lane 3) or p*Pst*I-G and pIE-1 (lane 4); or with 10 μg of total cell RNA from cells 6 h (lane 5) or 18 h (lane 6) after infection with AcNPV. (B) The 3′-end-labeled probe (lane 1) was a 2,657-bp *Bgl*II-*Xba*I fragment, specifically labeled at the *Xba*I site with the Klenow enzyme, hybridized with 20 μg of uninfected-cell RNA (lane 2) or 18-h infected-cell RNA (lane 3). (C) The 5′-end-labeled probe was a 1,388-bp *Bam*HI-*Xba*I probe, exclusively 5′ end labeled at the *Bam*HI site. The lane assignments are the same as those for panel A. (D) The 3′-end-labeled probe was a 2,328-bp *Bam*HI-*Xba*I fragment, specifically 3′ end labeled at the *Bam*HI site. The lane assignments are the same as those for panel B.

ORF-2. Transcripts terminating 285 bp downstream of the *Bgl*II site would stop within ORF-2.

Although all of the rightward transcripts contain ORF-2, it is not clear whether any of the transcripts could direct the synthesis of this protein. The late transcripts initiate within ORF-1, and several AUG codons are located between the 5′ ends of these transcripts and the initiation codon for ORF-2. The initiating AUG for ORF-2 is located 107 bp to the left of the *Bgl*II site. Although the conditions of the gel were sufficient to detect nuclease S1-protected fragments 100 nt or larger, we cannot rule out the possibility that a minor transcript was not detected.

The 5′ end of the leftward transcriptional unit was mapped with a 1,388-bp *Bam*HI-*Xba*I fragment exclusively labeled at the *Bam*HI site (Fig. 5B). A single protected fragment of 401 bp was observed after hybridization, S1 nuclease digestion, and denaturing polyacrylamide gel electrophoresis.

Two 3′ ends were mapped 988 and 747 bases downstream of the *Bam*HI site with a 2,329-bp *Bam*HI-*Xba*I fragment exclusively 3′ end labeled at the *Bam*HI site. Although these sites were not mapped to high resolution, the data indicate that the shorter transcripts terminate just downstream of the ORF-3 termination codon, while the longer transcripts terminate within ORF-2. Polyadenylation signals are located within 20 bp upstream of the putative polyadenylation sites.

Functional mapping of ORFs. To determine which of the three ORFs contained the *trans*-active factor(s), we cotransfected cells with pL2cat, pIE-1, and several subclones of pPGX (Fig. 6). Neither the 2,334-bp *Xba*I-*Bam*HI subclone, which contains ORF-1 and ORF-2, nor the 2,662-bp *Xba*I-*Bgl*II subclone, which contains ORF-3, was active alone. However, a mixture of these two clones *trans*-activated to the same extent as did the entire *Xba*I fragment, indicating that ORF-1 or ORF-2 was required in addition to ORF-3. Although it is unlikely that ORF-2 was transcribed in the transient assay, additional clones were constructed to distinguish between ORF-1 and ORF-2. The promoter and N terminus of ORF-1 were deleted from the *Xba*I-*Bam*HI subclone with exonuclease III; it is unclear what effect, if any, this deletion would have on ORF-2. To inactivate ORF-2 without affecting transcripts encoding ORF-1, we created a frameshift in the middle of ORF-2 by digestion of the *Xba*I-*Bam*HI subclone with *Bgl*II, repair with the Klenow enzyme, and religation. Neither of these constructs *trans*-activated alone (Fig. 6). However, when cotransfected with the *Xba*I-*Bgl*II subclone, the Δ*Bgl*II clone was active,

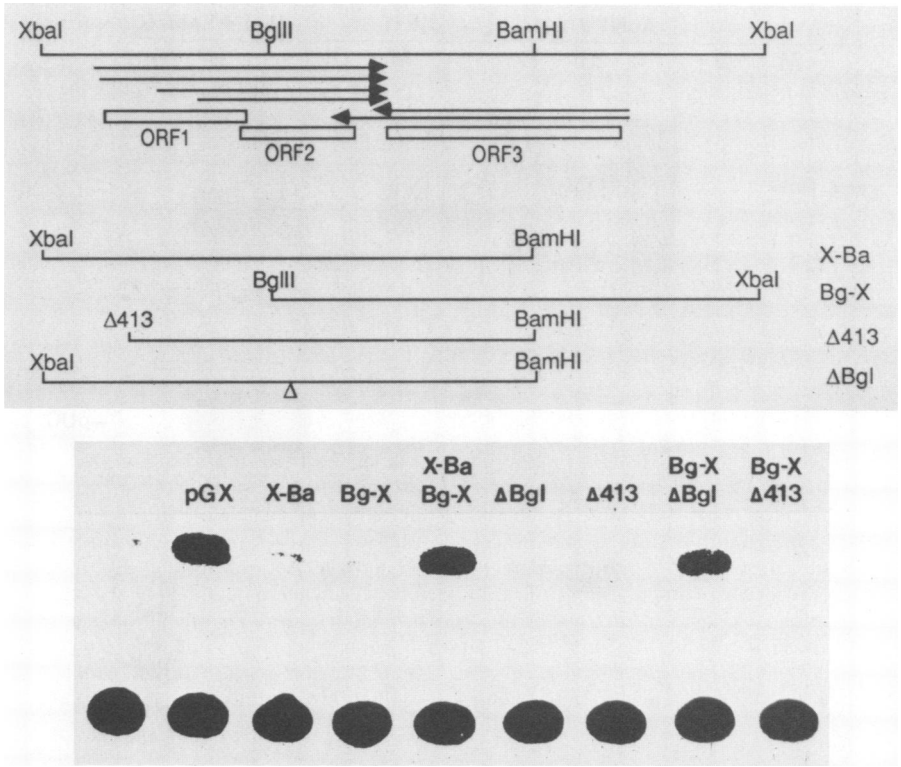

FIG. 6. Functional mapping of the three ORFs of the *Xba*I fragment of p*Pst*I-G. *S. frugiperda* cells were transfected with 1 μg each of pL2cat and pIE-1 and 1 μg of the indicated plasmids, and CAT activity was determined after 48 h. Clone X-Ba contains the leftmost 2,328-bp *Xba*I-*Bam*HI fragment. Clone Bg-X contains the rightmost 2,657-bp *Bgl*II-*Xba*I fragment. Clone Δ413 was derived from X-Ba by exonuclease III deletion of 413 bp of DNA adjacent to the *Xba*I site, removing the promoter and N-terminal portion of ORF-1. Clone ΔBgl, an ORF-2 frameshift mutant, was derived from X-Ba by digestion with *Bgl*II, followed by repair with the Klenow enzyme and religation. pGX = pPGX.

whereas the Δ413 clone was not. These data indicated that ORF-1 was required for *trans*-activation with ORF-3, while ORF-2 was dispensable.

DNA replication. In AcNPV-infected cells, late transcription is linked to DNA replication. To determine whether the expression of pL2cat was dependent upon DNA replication in the transient-expression assay system, we recovered plasmid DNAs from transfected cells and analyzed them by differential digestion with *Mbo*I or *Dpn*I (Fig. 7). The recovered plasmid DNAs were detected upon hybridization with pUC8 DNA radiolabeled with [^{32}P]dATP by nick translation. Plasmids pL2cat and pIE-1 each contain a single *Xba*I site, and fragments of 9.5 and 5.8 kb, respectively, were generated upon digestion with *Xba*I. Plasmid pPGX contains two *Xba*I sites, and fragments of 3.4 and 2.7 kb were generated; only the 2.7-kb vector band was detected by the pUC8 probe.

When pL2cat was cotransfected with AcNPV DNA, most of the recovered DNA was *Mbo*I sensitive and *Dpn*I resistant, indicating that the plasmid had undergone some replication. This result is not specific for pL2cat, as a similar result was observed with pUC8 DNA cotransfected with AcNPV DNA (data not shown). However, when pL2cat was cotransfected with pIE-1 or with pIE-1 and pPGX, the recovered plasmids were entirely *Mbo*I resistant and *Dpn*I sensitive.

DISCUSSION

This report indicates that at least one late gene of AcNPV can be expressed in a transient-expression assay system. Transcription was dependent upon IE-1, indicating that the IE-1 gene product may be required late in infection, in addition to its role in the *trans*-activation of delayed-early genes. There was also an absolute requirement for one of the *cis*-acting AcNPV enhancers. Expression of the late gene product was further increased by cotransfection of a 3.7-kb restriction fragment which maps to the *Pst*I G fragment of AcNPV DNA. Expression of the late gene apparently did not require replication of the plasmid DNA.

Immediate-early, delayed-early, and late promoters have characteristic behavior upon transfection of *S. frugiperda* cells. Immediate-early genes, such as IE-1, are active in the absence of additional viral factors (10). Several delayed-early genes have been shown to be dependent upon IE-1 for functional expression (12). Expression of the delayed-early 39K gene is enhanced by but is not dependent upon *cis*-linked viral enhancer elements (11). The results presented here indicate that late genes can be distinguished from delayed-early genes by their absolute dependence upon the presence of a *cis*-linked enhancer in addition to IE-1 for expression in the transient-expression assay.

In a productive viral infection, expression of late genes is linked to DNA replication (15). However, two reports have reached conflicting conclusions as to whether late gene expression is dependent upon DNA replication. In one report, the accumulation of late viral proteins was analyzed (6). After 44 h in the presence of cytosine arabinoside, empty capsids were formed and occlusion bodies were evident in infected cells. This result indicated that late genes are expressed in the absence of viral DNA replication, albeit at

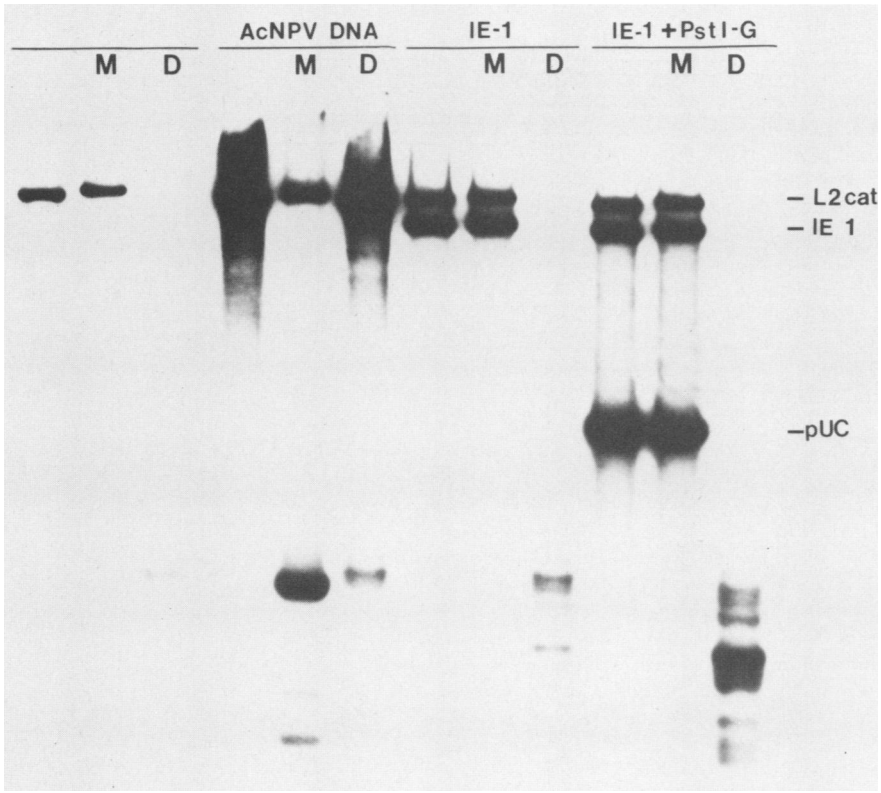

FIG. 7. Replication of plasmids in transfected cells. *S. frugiperda* cells were transfected with 1 μg of each of the indicated plasmids or viral DNA. After 48 h, low-molecular-weight DNA was isolated by the procedure of Hirt (13) and digested with *Xba*I and *Dpn*I (D) or *Mbo*I (M) The positions of the input plasmids are indicated on the right.

greatly reduced levels, presumably because of the lack of template amplification. In the other report (18), steady-state levels of late transcripts were examined. After 24 h in the presence of aphidicolin, late mRNA was not detected by Northern (RNA) blot hybridization. An explanation which would reconcile these two experiments is that late genes were transcribed in the absence of DNA synthesis but at a greatly reduced level which was not detected. However, sufficient mRNA was transcribed so that over a longer period of time, significant amounts of late proteins accumulated. Alternatively, it is possible that there are two classes of baculovirus late genes, as has been demonstrated in herpes simplex virus (19). Further characterization of baculovirus late genes is required to resolve this conflict.

Although our results demonstrate that at least one late gene was expressed in the absence of DNA replication, it is important to note that these results were obtained in a transient-expression assay system. Although this approach has been extensively used for the analysis of many viral genes, it is not clear how accurately a transient-expression assay mimics a viral infection.

Plasmid pL2cat underwent replication when cotransfected with viral DNA. Similar results have been obtained with all pUC8- and pBR322-derived plasmids that we have tested, including the pUC8 vector alone. Therefore, this result should not be interpreted to imply that pL2cat contains a viral origin of replication. This lack of specificity for DNA replication was somewhat unexpected. We have not yet extended these results to determine whether all DNAs replicate when cotransfected with viral DNA or whether the replication is limited to pUC-based plasmids. It is possible that pUC plasmids contain a sequence which resembles the viral origin of replication. To date, nothing is known concerning the viral sequences required for initiation of DNA replication.

At least a 10-fold-higher expression of pL2cat was observed in the presence of viral DNA than in the presence of pIE-1 and pPGX. Some of this difference can be ascribed to the replication of pL2cat in the presence of viral DNA, whereas pL2cat did not replicate in the defined system. Replication could increase gene expression in at least three different ways. One, an increase in the number of plasmids may linearly increase the number of transcripts. Two, replication of pL2cat may significantly alter the ability of the plasmid to be transcribed. Three, replication of the viral DNA may increase the amount of *trans*-active factors. In addition, it is possible that additional viral factors other than those on *Pst*I-G are required for late gene expression. One of those factors may be the virus-induced or virus-modified RNA polymerase II (7).

Preliminary results obtained by nuclease S1 analysis and with excess pL2cat probe indicated that the steady-state levels of pL2cat message were not increased by the presence of pPGX (data not shown). This result implies that the *Pst*I-G factors probably do not activate by increasing the rate of transcription or stability of the message. We intend to develop a cell-free translation system with *S. frugiperda* cells to test whether the *Pst*I-G factors increase the efficiency of translation of pL2cat.

Sequence analysis of ORF-3 revealed the presence of cysteine fingers (1), indicating that the gene product may interact with nucleic acid. It will be of interest to determine

whether the two regulatory proteins encoded by *Pst*I-G act in concert or whether their roles are entirely separate. The nucleotide sequences presented here should enable us to design experiments with antibodies to the two proteins to attempt to define their functions.

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