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Functional Mapping of a *trans*-Activating Gene Required for Expression of a Baculovirus Delayed-Early Gene

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The temporal regulation of an early gene of the baculovirus *Autographa californica* nuclear polyhedrosis virus was examined. We constructed a plasmid (plasmid 39CAT) in which the bacterial gene for chloramphenicol acetyltransferase was placed under the control of the promoter for the gene for a *A. californica* nuclear polyhedrosis virus 39,000-dalton protein (39K). A transient expression assay of plasmid 39CAT revealed that the 39K gene was expressed in infected cells but not in uninfected cells, indicating that the 39K gene should be classified as a delayed-early gene. The 39K promoter also efficiently directed the synthesis of chloramphenicol acetyltransferase when the plasmid was cotransfected with viral DNA which had been restricted with several restriction enzymes. To map the location of the gene(s) required for the synthesis of 39K, plasmid 39CAT was cotransfected with purified restriction fragments of *A. californica* nuclear polyhedrosis virus DNA. Fragments which mapped between 90.7 and 100.8 map units induced plasmid 39CAT. Plasmid p*Eco*RI-B, containing *Eco*RI fragment B (90 to 100 map units), activated plasmid 39CAT. Functional mapping of plasmid p*Eco*RI-B indicated that the essential region was located between 95.0 and 97.5 map units. The 5′ end of this gene was mapped, and the chloramphenicol acetyltransferase gene was inserted under the control of its promoter. Transient assay experiments indicated that the *trans*-acting regulatory gene was expressed in uninfected cells and is therefore an immediate-early gene. This gene was named IE-1.

The genome of *Autographa californica* nuclear polyhedrosis virus (AcNPV) is a double-stranded, supercoiled, circular DNA molecule of approximately 128 kilobase pairs. The temporal regulation of AcNPV gene expression is similar to that of most large DNA viruses. The expression of immediate-early (α) genes does not require prior viral protein synthesis, while delayed-early (β) genes apparently require an α gene product for expression (25). Synthesis of late (γ) genes is concomitant with, but may not be dependent upon, the onset of viral DNA synthesis (12, 25). Baculoviruses are unique in having a fourth temporal class of genes, the very late (δ) genes, which are associated with viral occlusion. The δ genes are highly expressed after the release of extracellular virus, while the synthesis of α, β, and γ gene products is repressed (9, 31, 47). Some genes are expressed in more than one temporal phase. For example, the gene for a protein having a molecular weight of 39,000 (39K) is the most abundantly expressed early gene and is also expressed late in infection (37, 41). Synthesis of 39K can be detected by 3 to 6 h postinfection, but it has not been determined whether the 39K gene is an α gene or a β gene.

AcNPV early transcripts map to a few dispersed regions of the genome, while late in infection most of the genome is transcribed (13, 14, 35). Overlapping nests of transcripts with coterminal 3′ or 5′ ends have been mapped in several regions of the genome (15, 27). One transcription unit was composed of five early and late RNAs with a common 3′ end. The smallest RNAs appeared early and were replaced in time by successively larger transcripts, suggesting that temporal regulation involves sequential activation of upstream promoters along with coordinate deactivation of downstream promoters (15). In contrast, the very late (δ) transcription units are composed of overlapping RNAs with coterminal 5′ ends. Splicing has not been detected in baculovirus transcripts (26).

Nothing is known about the mechanisms of regulation of temporal expression in baculoviruses. Two temperature-sensitive mutants blocked at an early stage of replication have been described previously (16, 33). The mutations were mapped to two different regions of the genome, but the identity and normal function of the mutated genes are unknown.

We identified and functionally mapped a gene which regulates temporal expression in AcNPV. The bacterial gene for chloramphenicol acetyltransferase (CAT) was cloned under the control of the 39K gene. The resulting construction, plasmid 39CAT, was active in infected cells but not in uninfected cells, indicating that the 39K gene is a delayed-early gene. By using cotransfection of plasmid 39CAT and purified restriction fragments of viral DNA or cloned viral DNA fragments, we identified a viral immediate-early gene which *trans*-activates 39K gene expression.

MATERIALS AND METHODS

Cells and virus. *Spodoptera frugiperda* IPL-21 AEIII cells obtained from S. Weiss (Cetus Corp., Emeryville, Calif.) were used in transfection experiments. These cells were propagated in IPL-41 monolayer medium (46) containing 10% fetal bovine serum (KC Biologicals, Lenexa, Kans.). For preparation of viral DNA and infected cell RNA, *S. frugiperda* clone 9 spinner cultures were grown in TNMFH medium (20) supplemented with 10% fetal calf serum. The cells were concentrated by centrifugation at 1,000 × *g* for 5 min, suspended at a density of 10^7 cells per ml, and inoculated with extracellular virus at a multiplicity of 20 PFU per cell. After a 1-h adsorption period at room temperature, the cells

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were centrifuged as described above and suspended in fresh TNMFH medium at a density of 10^6 cells per ml. After 48 h, the cells were centrifuged at 1,000 × *g*. The supernatant was adjusted to 10% polyethylene glycol and 0.1 M NaCl, and the preparation was gently stirred at 4°C for 1 h. The virus precipitate was collected by centrifugation at 10,000 × *g* for 10 min. The pellet was gently suspended in 5 ml of 10 mM Tris (pH 7.5)–1 mM EDTA and then mixed with an equal volume of 20% polyethylene glycol to 0.2 M NaCl. After 1 h at 4°C, the precipitated virus was centrifuged and suspended as described above. The virus suspension was adjusted to 1% sodium dodecyl sulfate and extracted with phenol.

RNA isolation. Cells were infected as described above. At 6 or 18 h postinfection the cells were centrifuged, washed once in phosphate-buffered saline, and suspended in five times the packed cell volume of 6 M guanidinium isothiocyanate–5 mM sodium citrate–0.1 M β-mercaptoethanol–0.5% Sarkosyl. Then 1 g of CsCl per ml of supernatant was added, the mixture was layered onto 1.2 ml of 5.7 M CsCl, and the preparation was centrifuged at 30,000 rpm in a type SW60 rotor for 16 h. The RNA pellet was suspended and extracted as described previously (30).

Recombinant plasmids. The 39CAT plasmids were constructed in several steps. First, the termination and polyadenylation sequences for the polyhedrin gene were excised from plasmid pAc373 (G. E. Smith, G. Ju, B. L. Erickson, J. Moschera, H.-W. Lahm, R. Chizzonite, and M. D. Summers, Proc. Natl. Acad. Sci. USA, in press) with *Bam*HI and *Eco*RI. The resulting 3-kilobase (kb) fragment was cloned into the *Bam*HI and *Eco*RI sites of pUC8. The coding sequences for the bacterial CAT gene were derived from plasmid pCAT (28). The CAT gene was excised with *Bam*HI and cloned into the *Bam*HI site adjacent to the polyhedrin sequences. The proper orientation of the CAT gene was determined by *Eco*RI digestion. This plasmid had only one unique cloning site upstream from the CAT gene. A second unique site was created by removing the *Sal*I site in the polyhedrin sequences by successively performing the following steps: partial *Sal*I digestion, purification of full-length DNA, Klenow repair, blunt end ligation, and transformation of *Escherichia coli* strain JM83 cells. Plasmid pAc3075, which had a single *Sal*I site in the multiple cloning region, was selected. The 5′ end of the 39K message has been mapped to a site ca. 220 base pairs (bp) upstream from a *Pvu*II site in *Pst*I fragment K of the AcNPV genome (J. Vlak, no. 23, Abstr. Soc. Invertebr. Pathol. 1985). To remove the coding sequences between the promoter and the *Pvu*II site, plasmid p*Pst*I-K (20 μg) was digested with *Pvu*II and incubated with 0.5 U of *Bal* 31 buffer at 30°C (30). At 5-min intervals, 10-μl portions were removed and adjusted to 50 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and the digested ends were made blunt by incubation with the Klenow fragment of DNA polymerase I.

The digested DNAs were extracted with phenol, precipitated with ethanol, suspended, and digested with *Pst*I. After electrophoresis in 1% agarose, fragments ca. 750 to 1,050 bp long were eluted and cloned into the *Pst*I and *Sal*I (repaired) sites of pAc3075. Seven plasmids containing different-length promoter sequences were tested by transfecting AcNPV-infected *S. frugiperda* cells and assaying for CAT activity. The plasmid yielding the highest activity (plasmid 39CAT-4) was chosen for further study.

The *Hin*dIII and *Eco*RI plasmids were constructed by cloning the appropriate restriction fragments in the *Hin*dIII and *Eco*RI sites of pUC8. Recombinant plasmids were propagated in *E. coli* strains JM83 and TB1. Plasmid DNA was isolated by the method of Holmes and Quigley (21) and purified by centrifugation in cesium chloride-ethidium bromide gradients.

Transfections and CAT assays. Cells were transfected by a modification of the procedure of Graham and van der Eb (18). Subconfluent monolayers of *S. frugiperda* IPL-21 AEIII cells were harvested by scraping with a rubber policeman and suspended by gentle pipetting. The cells were seeded at a density of 10^6 cells per 60-mm dish. The cells were allowed to attach for 1 h, and then the medium was replaced with 0.5 ml of fresh medium. DNAs to be transfected were diluted in 0.5 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (25 mM HEPES, pH 7.1, 140 mM NaCl) containing 125 mM $CaCl_2$. The unprecipitated DNAs were added dropwise to the cell monolayers. Calcium phosphate precipitates formed due to the $CaCl_2$ in the HEPES-buffered saline and the phosphate in the medium. Each transfection mixture contained 2 μg of 39CAT-4 and 2 μg of viral or plasmid DNA unless otherwise noted. For transfections of purified gel fragments, 10 μg of viral DNA was digested with a restriction enzyme, and fragments were separated by electrophoresis through 0.6% low-melting-temperature agarose. The purified fragments were eluted by heating the preparation at 65°C for 15 min, followed by phenol extraction and ethanol precipitation. The amount of a DNA fragment purified from 10 μg of digested DNA was added to each dish. For transfections in which the total amount of transfected DNA was less than 4 μg, calf thymus DNA was added to bring the amount up to 4 μg.

Cells were harvested 24 h after transfection, and CAT assays were performed as described previously (18), except that cells were broken by three cycles of freezing and thawing instead of sonication and each assay mixture contained only 0.2 μCi of [^{14}C]chloramphenicol.

DNA sequencing. The 39CAT plasmids were digested with *Pst*I and *Bam*HI, and the promoter fragments were cloned into the *Pst*I and *Bam*HI sites of M13mp9 (32). Single-stranded recombinant phage was purified and sequenced by the dideoxy chain termination method (36). The sequence of the opposite strand was determined by cloning and sequencing the appropriate *Hpa*II restriction fragments of *Pst*I-K in the *Acc*I site of mp9.

S1 nuclease mapping of AcNPV DNA. The 5′ ends of the 39K and IE-1 transcripts were mapped by the S1 procedure of Berk and Sharp (6), as modified by Weaver and Weissman (45); 5′-end-labeled probes were generated by digesting p*Pst*I-K and pIE-1 with *Cla*II and *Hin*fI, respectively. The 5′ ends were dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and radiolabeled with T4 polynucleotide kinase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and [γ-^{32}P]ATP (4,000 Ci/mmol). The DNAs were then digested with *Pst*I (p*Pst*I-K) or *Bam*HI (IE-1), and the appropriate restriction fragments were purified by agarose gel electrophoresis. DNA probes (0.1 μg) were hybridized to 10 μg of total cellular RNA in a solution containing 80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethansulfonic acid)] (pH 6.4), 0.4 M NaCl, and 1 mM EDTA for 4 h at 42°C. Samples were diluted 10-fold into S1 buffer (30 mM sodium acetate, pH 4.6, 4.5 mM $ZnSo_4$, 0.25 M NaCl, 20μg of single-stranded DNA per ml) containing 100 U of S1 nuclease per ml and incubated at 37°C for 30 min. Digestion was terminated by adding 75 μl of S1 stop buffer (2.5 M ammonium acetate, 0.1 M EDTA). The S1 hybrids were

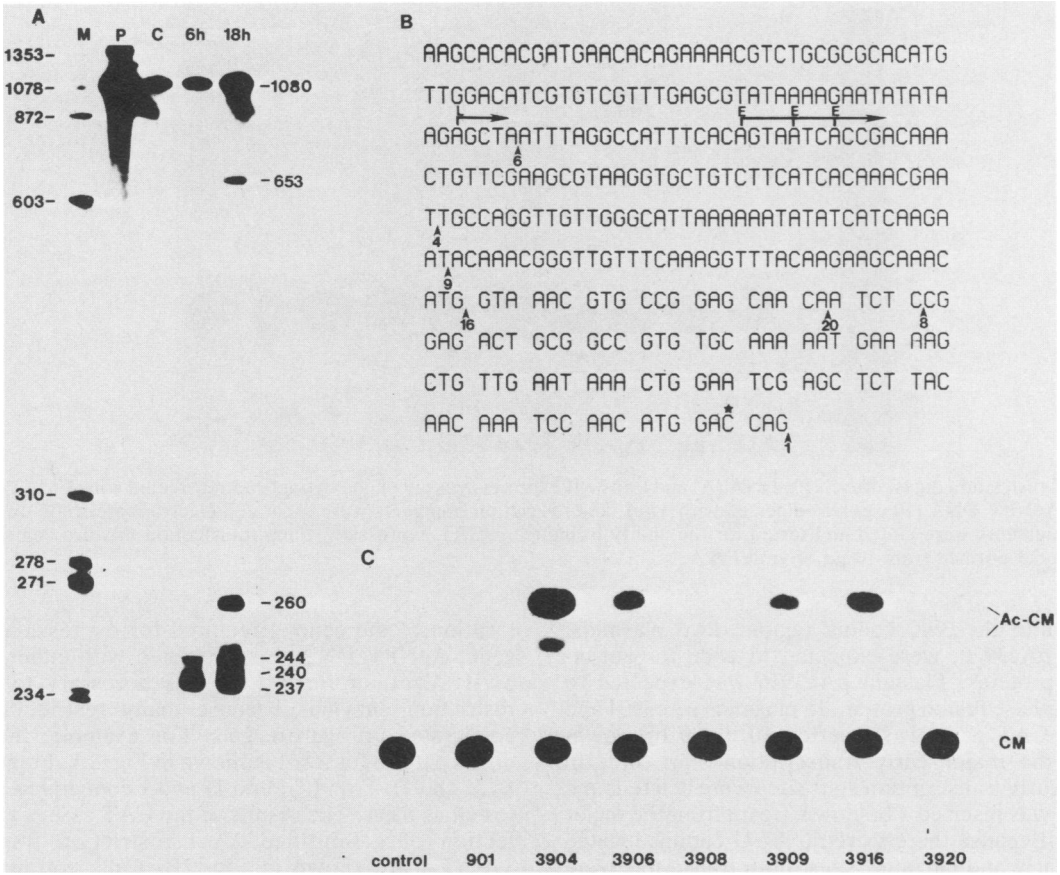

FIG. 1. S1 nuclease mapping of the 5′ ends of the 39K gene (A), the nucleotide sequence of the 5′ end of the gene (B), and transient expression of chimeric 39CAT plasmids (C). (A) Mapping of the 5′ ends of viral transcripts isolated 6 and 18 h postinfection or from control, uninfected cells (lane C). Total cellular RNA (10 μg) was hybridized with 0.1 μg of the 1.07-kb *Pst*I-*Cla*II fragment (the position of the label in the *Cla*II site is indicated by an asterisk in panel B). The size of the S1-resistant fragment was determined by electrophoresis on a 7 M urea–5% polyacrylamide gel. An autoradiograph of the gel is shown. The sizes of the molecular weight markers (ϕX174 *Hae*III fragments) are indicated on the left, and the sizes of the S1-resistant DNAs are indicated on the right. Lane P contained the 5′-end-labeled DNA probe before digestion. Lane M contained the molecular weight markers. (B) Sequence of the 5′ end of the 39K gene. The numbered arrowheads indicate the extents of the *Bal* 31 deletions for the plasmids indicated. The arrows marked with E and L indicate the early and late transcriptional start sites, respectively. The position of the 5′ end label used for S1 mapping is indicated by an asterisk. (C) Transient expression of 39CAT plasmids. *S. frugiperda* cells were infected with AcNPV 1 h prior to transfection with 2-μg portions of the plasmids indicated at the bottom. At 24 h posttransfection, cell extracts were prepared and analyzed for CAT activity. An autoradiograph of the thin-layer chromatogram is shown. The positions of input unacetylated chloramphenicol (CM) and acetylated products (Ac-CM) are indicated.

precipitated with ethanol, denatured, and analyzed by electrophoresis on 5% polyacrylamide–7 M urea–89 mM Tris hydrochloride (pH 8.3)–89 mM boric acid–2 mM EDTA gels. Size standards were derived from 3′-end-labeled *Hae*III digests of ϕX174 DNA (30).

RESULTS

Temporal regulation of 39K. The 39K gene has been mapped to *Eco*RI fragment J of AcNPV DNA (14, 41) (see Fig. 3 for the location of the 39K gene). The 5′ end is located approximately 220 bp upstream from a *Pvu*II site in AcNPV DNA *Pst*I fragment K (Vlak, no. 23, Abstr. Soc. Invertebr. Pathol. 1985). Because baculovirus genes apparently are not spliced (26), we used high-resolution S1 mapping to determine the precise location of the 5′ end of the 39K gene. The 1,080-bp *Pst*I-*Cla*II fragment of *Pst*I-K was labeled at the *Cla*II site and hybridized to total cellular RNA isolated at 6 and 18 h postinfection (Fig. 1A). The following four S1-resistant DNA fragments were resolved when the DNA probe was hybridized to 6-h RNA: a major band at 237 nucleotides, two minor bands at 240 and 244 nucleotides, and a very faint band at 653 nucleotides. When the probe was hybridized to 18-h RNA, the same four S1-resistant bands were observed. In addition, another major band at 260 bp was present.

In order to clone the CAT gene under the control of the 39K promoter, *Bal* 31 was used to remove the coding sequences located between the promoter and the *Pvu*II site. The approximate extent of the *Bal* 31 deletions was determined by restriction digestion and agarose gel electrophoresis. Six plasmids with promoter fragments of different lengths were sequenced to determine the precise point at which the CAT gene was inserted (Fig. 1B). Plasmid pAc3901 contained the complete 1,080-bp *Pst*I-*Pvu*II fragment. Assuming that the first AUG sequence downstream from the transcriptional start sites was the initiating AUG sequence, pAc3901 would be expected to code for an in-phase fusion protein. In three of the deletions, the CAT gene

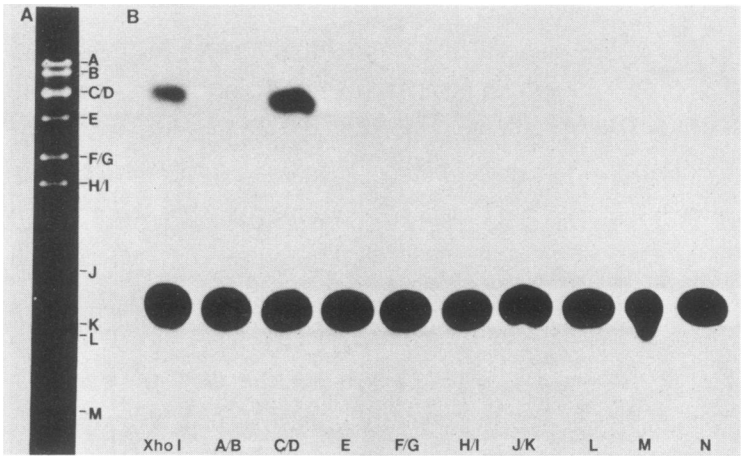

FIG. 2. *Xho*I restriction digest of AcNPV DNA (A) and transient expression assay of cell extracts cotransfected with 39CAT-4 and purified fragments (B). AcNPV DNA (10 μg) was digested with *Xho*I, and restriction fragments were separated electrophoretically on 0.6% agarose gels. Purified fragments were eluted and tested for the ability to induce 39CAT expression. Each transfection mixture contained the total amount of fragment purified from 10 μg of viral DNA.

was inserted into the 39K coding region. Two plasmids, pAc3908 and pAc3920, were expected to code for out-of-phase fusion proteins. Plasmid pAc3916 was expected to code for an in-phase fusion protein. In plasmids pAc3904 and pAc3909, the CAT gene was inserted 50 or 90 bp downstream from the major early transcription start site. In pAc3906, the early transcription start sites were deleted, and the CAT gene was inserted 4 bp downstream from the major late start site. Because there were no AUG codons located between the early and late start sites, both transcripts were expected to code for the same protein.

To examine the temporal regulation of the 39K promoter, *S. frugiperda* cells were infected with AcNPV and 1 h later were transfected with 2-μg portions of the various 39CAT plasmids. After 24 h, cellular extracts were prepared, and CAT assays were performed (Fig. 1C). As expected, there was no CAT activity detected in the two plasmids in which the CAT gene was inserted out of phase. The in-phase fusion protein produced by pAc3916 was active. These results indicate that the assignment of the initiating AUG codon was correct. Plasmid pAc3901, which encoded a larger fusion protein, had little activity. The two plasmids in which the CAT gene was inserted in the 5′ leader of the early transcripts were active in directing the synthesis of CAT. The late promoter also efficiently directed the synthesis of CAT, as evidenced by the activity observed in cells cotransfected with pAc3906.

The seven plasmids which we constructed were also used to transfect *S. frugiperda* cells that had not been infected prior to transfection. No CAT activity was observed with any of the plasmids in extracts of uninfected cells (data not shown), indicating that the 39K promoter was not transcriptionally active in uninfected cells. These results are consistent with a classification of delayed early for the 39K gene, because a viral function is apparently required for expression of the 39K gene. Plasmid pAc3904 (= plasmid 39CAT-4) was chosen for further study.

Functional mapping of the gene required for 39K expression. The 39K promoter was efficiently induced when uninfected cells were cotransfected with 2 μg of 39CAT-4 and 2 μg of viral DNA. The promoter was also stimulated by viral DNA that was digested with several different restriction enzymes prior to transfection (data not shown). To map the location of the gene(s) required for expression of the 39K gene, AcNPV DNA was restricted with either *Kpn*I, *Sst*I, *Sst*II, *Xho*I, or *Bam*HI. It was necessary to use several restriction enzymes because many restriction fragments comigrated on agarose gels. For example, the restriction digest pattern for *Xho*I is shown in Fig. 2A. Fragments A and B, C and D, F and G, and H and I comigrated and so were tested as pairs. The results of the CAT assays after cotransfection of gel-purified *Xho*I restriction fragments and 39CAT-4 are shown in Fig. 2B. Cells cotransfected with 39CAT-4 and *Xho*I fragments C and D synthesized CAT, indicating that the gene(s) needed to activate the 39K gene are located on either one or both of these fragments. The other fragments which induced CAT activity were *Kpn*I fragments A, B, and C, *Sac*II fragments C and D, *Sac*I fragments A and B, and *Bam*HI fragment B (data not shown). By constructing a consensus map from several digests, it was possible to eliminate all but one fragment from each digest (Fig. 3). Our combined data indicated that the region from 90.7 to 0.8 map units (m.u.) contained the gene required for expression of the 39K gene. The consensus region was defined by the left end of *Xho*I fragment C and the right end of *Sst*II fragment D. The circular AcNPV genome is presented as linear by convention, with the 0 position of the map at the left end of *Eco*RI fragment I (40).

*Eco*RI fragment B (90 to 100 m.u.) covered most of the consensus region indicated in Fig. 3. This fragment was cloned into the *Eco*RI site of pUC8 and tested in the transient assay system for its ability to induce 39CAT expression. CAT was efficiently expressed in cells transfected with 39CAT-4 and p*Eco*RI-B (data not shown). To further define the location of the gene required for expression of the 39K gene, a detailed restriction map of p*Eco*RI-B was generated (Fig. 4A). A series of deletions in *Eco*RI fragment B was constructed by digestion and religation with *Sal*I, *Cla*I, *Eco*RV, *Xba*I, or *Bgl*II (Fig. 4B). In addition, plasmids containing *Hin*dIII fragments G and F were cloned and tested. All of the activity was located in *Hin*dIII fragment G. Deletion of the *Xba*I fragment between 96.7 and 97.5 m.u. did not eliminate CAT activity, indicating that the gene was located to the left of 96.7 m.u. on the physical map. Deletion of the region between the *Sal*I site in the multiple cloning site and the *Sal*I site at 92.2 m.u. had no

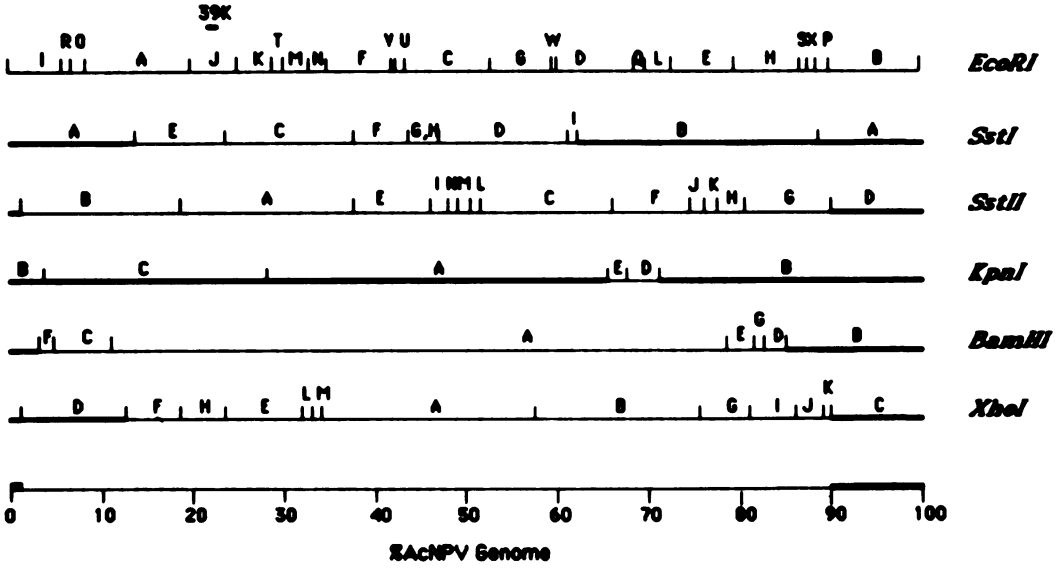

FIG. 3. Consensus map of the DNA fragments which activate 39CAT-4. Fragments which were positive in the transient expression assay are indicated by heavy lines. The consensus region is shown as a shaded line. The circular AcNPV genome was linearized at *Eco*RI fragment I by convention. The genomic location of the 39K gene is indicated.

effect, indicating that the gene was located to the right of 92.2 m.u.

Two of the deletions (the *Cla*I and *Eco*RV deletions) did not activate 39CAT expression. The *Cla*I fragment (95 to 99.8 m.u.) and the *Eco*RV fragment (95.3 to 97.8 m.u.) were cloned into the *Acc*I or *Hin*cII sites of pUC8 and tested for their ability to activate 39CAT-4 in the transient assay system. The plasmid containing the *Cla*I fragment induced CAT, but the plasmid containing the *Eco*RV fragment did not (data not shown). This result indicated that one end of the gene required for expression of the 39K gene was located between the leftmost *Cla*I and *Eco*RV sites and the other end was located to the left of the *Xba*I site at 97.5 m.u. Because most AcNPV genes are transcribed from left to right (15, 27) on the linear map (clockwise on the circular map), we expected that the end located between the *Cla*I and *Eco*RV sites was the 5′ end.

Plasmid p*Eco*RI-B was cotransfected with pAc3916, pAc3909, and pAc3906 to determine whether these CAT plasmids were activated by the same gene as 39CAT-4 (data not shown). CAT was expressed in cells transfected with pAc3916, the plasmid producing an in-phase fusion protein, and in cells transfected with pAc3909, in which the CAT gene was inserted in the leader downstream from 39CAT-4. No CAT activity was detected in cells transfected with pAc3906, in which the 5′ end of the early transcript was deleted, indicating that the early and late transcripts were differentially regulated.

Temporal regulation of IE-1. The *Cla*I fragment of *Eco*RI-B was cloned into the *Acc*I site of pUC8 in both orientations. In one orientation, digestion with *Hin*dIII followed by religation removed the sequences to the right of 96.9 m.u. The resulting plasmid, which contained 3 kb of viral DNA, was designated pIE-1 (for immediate-early gene 1). To confirm

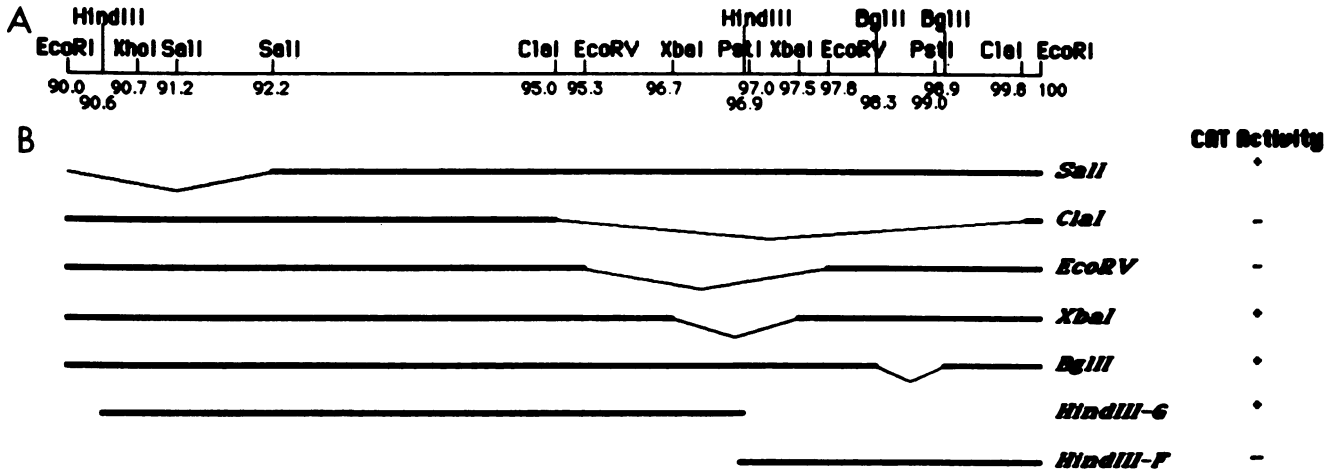

FIG. 4. Detailed restriction map of *Eco*RI-B (A) and construction of *Eco*RI fragment B subclones (B). Five subclones were constructed by restriction with *Sal*I, *Cla*I, *Eco*RV, *Xba*I, and *Bgl*II, followed by religation. The regions of *Eco*RI fragment B deleted by digestion are indicated by diagonal lines. p*Hin*dIII-G and p*Hin*dIII-F contained the restriction fragments indicated. The results of a transient expression assay for each clone are shown by a plus sign (induced 39CAT) or a minus sign (no CAT activity).

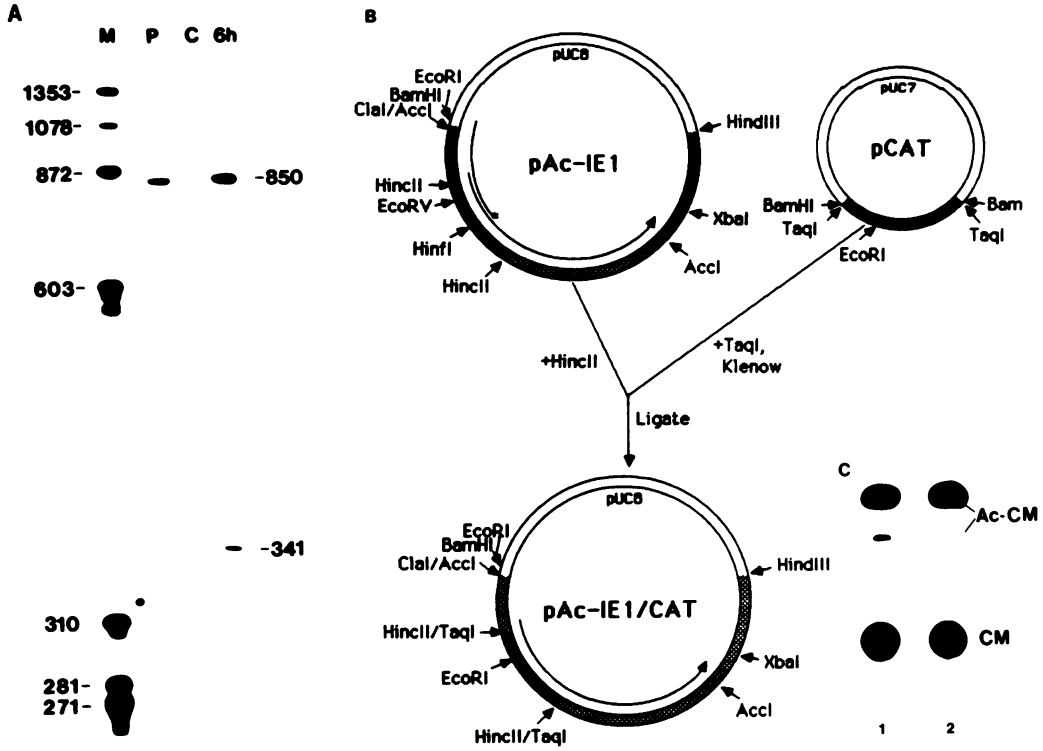

FIG. 5. S1 mapping of the 5′ end of the IE-1 gene (A), construction of an IE-1–CAT plasmid (B), and expression of the plasmid in infected and uninfected cells (c). (A) S1 nuclease mapping of the 5′ end of the IE-1 gene. Total cell RNAs (10 μg) from cells infected for 6 h or control, uninfected (c) cells were hybridized with 100-pg portions of an 850-bp *Bam*HI-*Hin*fI probe (lane P), which was specifically radiolabeled at the *Hin*fI site. S1-resistant hybrids were denatured and analyzed on 7 M urea–5% polyacrylamide gels. The sizes of the ϕX174 molecular weight standards (lane M) are indicated on the left, and the sizes of the S1-resistant fragments are indicated on the right. (B) Plasmid IE-1 was digested with *Hin*cII, and a 770-bp *Taq*I fragment of pCAT which was made blunt ended by Klenow repair treatment was cloned into the *Hin*cII site. The location and direction of transcription of the IE-1 transcript are indicated by an arrow. The location of the probe for S1 mapping is indicated by an asterisk. (C) AcNPV-infected cells (lane 2) and uninfected cells (lane 1) were transfected with 2 μg of IE-1–CAT. After 24 h, the CAT activity in cellular extracts was assayed. CM, Input unacetylated chloramphenicol; Ac-Cm, acetylated chloramphenicol products.

tht the temporal regulation of this gene was characteristic of an immediate-early gene, a chimeric IE-1–CAT plasmid was constructed. The 5′ end of the IE-1 gene was located by S1 mapping, using an 850-bp *Bam*HI-*Hin*fI probe exclusively labeled at the *Hin*fI site. Figure 5B shows the map location of the probe. The 5′ end of the IE-1 gene was located 341 bp upstream from the *Hin*fI site. A *Hin*cII site was mapped approximately 20 bp downstream from the transcription start site. The CAT gene was cloned into this *Hin*cII site after *Taq*I digestion of pCAT, followed by Klenow repair of the ends (Fig. 5B). The resulting plasmid, pIE-1/CAT, was used to transfect *S. frugiperda* cells. As shown in Fig. 5, CAT was expressed in uninfected cells, as well as in infected cells, indicating that the gene which *trans*-activated 39K gene expression was an immediate-early gene by definition because prior viral protein synthesis was not required for expression.

Construction of a plasmid containing 39CAT and IE-1. A plasmid containing 38CAT and the gene required for its expression was constructed. A *Cla*I subclone of p*Eco*RI-B, cloned in the opposite orientation as pIE-1 relative to multiple cloning site, was digested with *Pst*I. *Pst*I cut in the multiple cloning site and at 97.0 m.u. The resulting 3-kb fragment was cloned into the *Pst*I site of 39CAT-4 (Fig. 6A). The CAT activity induced by the 39CAT–IE-1 plasmid was equivalent to the activity induced by cotransfection of the two separate plasmids (Fig. 6B).

DISCUSSION

We examined the temporal regulation of an early gene of the baculovirus AcNPV. A protein having a molecular weight of 39,000 (39K) is the most abundantly expressed early protein and is also synthesized late in infection. To study the temporal regulation of this protein, we cloned the bacterial CAT gene under the control of the 39K promoter and assayed for the induction of the CAT gene to monitor the expression of the 39K gene.

Because baculovirus genes apparently are not spliced (26), we used S1 nuclease to map the 5′ ends of 39K transcripts isolated at 6 and 18 h postinfection. With RNA isolated 6 h postinfection, a major transcriptional start site was mapped 124 nucleotides upstream from the initiating AUG codon of the 39K gene. Two minor start sites were detected 3 and 7 nucleotides upstream from the major site. With RNA isolated 18 h postinfection, a major late transcriptional start site was mapped 23 nucleotides upstream from the major early site. The sequence of the 39K promoter indicates that the early and late transcripts should code for the same protein, because there are no initiating AUG codons located between the two sites. Expression of the CAT gene under the control of both promoters indicates that early and late transcripts direct the synthesis of 39K. The use of dual promoters for early and late genes has been described previously for genes of vaccinia virus (10), and simian virus 40 (43), and for a

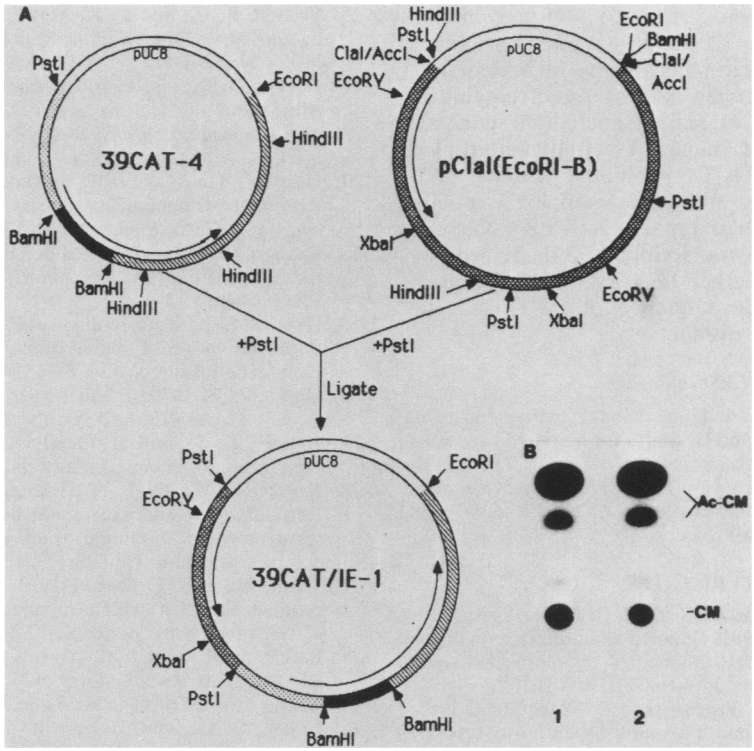

FIG. 6. Construction of a single clone containing 39CAT and the gene required for its expression (A) and 39CAT expression in transfected cells (B). (A) A plasmid containing the *Cla*I fragment of *Eco*RI fragment B (opposite orientation of the plasmid mentioned above) was digested with *Pst*I, which cut in the multiple cloning site and in the insertion. The resulting 3.0-kb fragment was cloned into the *Pst*I site of 39CAT-4. (B) Results of a CAT assay done with cells cotransfected with 39CAT-4 and p*Cla*I(*Eco*RI-B) (lane 1) or cells transfected with plasmid 39CAT-4/IE-1 alone (lane 2). CM, Input unacetylated chloramphenicol; Ac-Cm, acetylated chloramphenicol products.

developmentally regulated gene of *Drosophila melanogaster* (4).

The 5′ end of another minor early transcript was detected approximately 400 bp upstream from the 39K gene. This transcript is probably not involved in 39K gene expression, because there are numerous AUG codons upstream from the 39K gene. Overlapping unspliced transcripts have been described previously in several other regions of the AcNPV genome (15, 27), as well as in vaccinia virus (3, 29) and herpes simplex virus (1, 42). Friesen and Miller (15) described a 3′ AcNPV coterminal nest of overlapping transcripts in which the smaller RNAs were synthesized early and the larger RNAs were synthesized later. These authors proposed a model in which upstream promoters are sequentially activated, accompanied by deactivation of downstream promoters.

We transfected *S. frugiperda* cells with seven plasmids which had the CAT gene inserted at various positions in the 39K gene 5′ nontranslated leader and coding sequences. To confirm that the assignment of the initiating AUG codon was correct, we tested in-phase and out-of-phase fusion proteins. CAT activity was detected in AcNPV-infected cells but not in uninfected cells, indicating that the 39K gene requires a viral function and is therefore not an immediate-early gene. Because expression of the 39K gene can be detected as early as 3 to 6 h postinfection and after DNA synthesis (37), the 39K gene should be classified as a delayed-early/late gene.

The gene which *trans*-activates 39K gene expression was functionally mapped by cotransfecting cells with 39CAT-4 and fragments of viral DNA, followed by an assay for CAT activity. The gene required for 39K gene expression was designated IE-1 (for immediate-early gene 1). IE-1 was demonstrated to be an immediate-early gene by cloning the CAT gene under the control of the IE-1 promoter. IE-1–CAT was efficiently expressed in uninfected cells as well as in infected cells. IE-1 did not stimulate pAC3906, the plasmid in which the early promoter was deleted and the CAT gene was apparently under the control of the late promoter. This result indicates that IE-1 *trans*-activates the delayed early half of the tandem promoter and that the late half is differentially regulated. It will be of interest to determine whether IE-1 *trans*-activates all delayed-early genes or whether other viral factors are required.

Functional mapping with CAT was used previously to map the *trans*-activating gene of human T-cell lymphtropic viruses (2, 38, 39). A similar procedure with the thymidine kinase gene was used to map the structural protein which activates herpes simplex virus ICP4 (8). Traditionally, regulatory genes have been identified by using temperature-sensitive mutants. Cotransfection with CAT plasmids should be useful for functional mapping of viral genomes when libraries of temperature-sensitive mutants are not available. The mutation of an early temperature-sensitive mutant of AcNPV, blocked at an early stage of replication, was mapped to 90.7 to 1.9 m.u. (33), the same region of the genome as IE-1, indicating that the two procedures are complimentary. We intend to use the cotransfection mapping procedure to identify the gene(s) required for expression of pAc3906.

The AcNPV IE-1 gene product has a *trans*-acting regulatory role in 39K gene expression. *trans*-Acting regulatory genes have been described in adenoviruses (5, 23), simian

virus 40 (7, 24), herpes simplex virus (11, 44), and human T-cell lymphotrophic viruses (2, 38, 39). While these genes are functionally similar, their mechanisms of action may differ. Simian virus 40 T-antigen acts by direct binding to specific DNA sequences (7, 24), and the nucleotide sequence of the *trans*-acting gene of human T-cell lymphotrophic viruses is consistent with a DNA-binding activity (39). However, adenovirus type 5 and herpes simplex virus appear to act indirectly (19, 22) and may inactivate a host cell factor which inhibits viral transcription (34). It will be interesting to determine whether IE-1 acts at the level of transcription, the nucleotide sequence of IE-1, and the cellular location of the gene product.

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