JOURNAL OF VIROLOGY, Feb. 1989, p. 493–503
0022-538X/89/020493-11$02.00/0
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Vol. 63, No. 2

Molecular Analysis of the Transcriptional Regulatory Region of an Early Baculovirus Gene†

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Received 26 July 1988/Accepted 6 October 1988

Transcription of the gene encoding a 35,000-molecular-weight protein (35K protein) from the *Eco*RI-S region (86.8 to 87.8 map units) of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) occurs early in infection and declines later. The region promoting the gene for the 35K protein, extending from 426 base pairs (bp) upstream to 12 bp downstream from the RNA start site, was linked to the bacterial chloramphenicol acetyltransferase gene (CAT) for analysis. CAT expression was monitored in cells that were transfected with plasmids containing the promoter-CAT fusion as well as cells infected with recombinant viruses containing the chimeric gene inserted into the AcMNPV genome. Mapping of the 5′ ends of CAT-specific RNAs indicated that transcription initiated from the proper sites in both assays; moreover, the promoter fragment retained its early activity, despite an alternate location in the viral genome. The 5′ boundary of upstream regulatory sequences was determined by constructing deletions of the promoter fragment extending toward the early RNA start site (position +1). In transient assays, a gradual reduction in CAT expression occurred as sequences from positions −426 to −31 were removed. In contrast, promoter deletions from positions −426 to −155 in recombinant viruses exhibited no effect on CAT expression, whereas deletions to position −55 abolished early expression but had no effect on late expression. Late CAT expression was eliminated when deletions to position −4 removed part of the late RNA start site. DNA signals potentiating early transcription were therefore located upstream (between positions −155 and −55) from those involved in late transcription of the gene encoding the 35K protein. Potential consensus sequences for early and late regulatory elements were identified.

Multiplication of *Autographa californica* nuclear polyhedrosis virus (AcMNPV), the prototype of the insect baculoviruses, is distinguished by the production of two morphologically and temporally distinct forms of infectious progeny: extracellular nonoccluded virus and polyhedral occluded virus. Regulation of this process is complex, in that it involves the sequential and coordinated expression of early, late, and very late (occlusion-specific) genes. The timing and relative levels of gene expression from the double-stranded, circular DNA genome (128 kilobase pairs [kbp]) are controlled to a large extent at the transcriptional level. Host RNA polymerase II mediates early viral transcription, whereas a viral-induced RNA polymerase that is resistant to α-amanitin is responsible for a majority of viral transcription late in infection (11). AcMNPV RNA transcripts are capped and polyadenylated, but to date only one transcript, that for the immediate-early gene IE1, is known to be spliced (6). IE1 is involved in transcriptional regulation through the *trans*-activation of a number of early viral genes and at least one late viral promoter (16, 18). IE1-mediated stimulation can also be augmented in transient expression assays by the product of another immediate early gene, IE-N (4), as well as the *cis*-acting homologous region (*hr*) enhancer elements dispersed throughout the AcMNPV genome (17).

Transcriptional regulation of large DNA viruses involves the interaction of *cis*-acting regulatory sequences (promoters and enhancers), with *trans*-acting host and viral proteins making up the transcription apparatus. In the case of the strong promoter for the late AcMNPV gene encoding polyhedrin, the major structural protein of occluded virus particles, a relatively small AT-rich sequence (TAAGTATT) making up the RNA initiation site, is essential for regulated and high-level expression (25, 30, 31a). This regulatory motif is highly conserved between baculovirus polyhedrin promoters (for reviews, see references 28 and 32). Moreover, the RNA initiation sites of many AcMNPV late genes closely resemble the motif, suggesting that it also plays a central role in coordinating late baculovirus gene expression.

Unlike AcMNPV late promoters, little is known concerning the organization of early promoters or the mechanisms used to differentiate between early and late viral genes. To investigate the *cis*-acting DNA signals that are required for early expression, we focused on the transcriptional regulation of an early AcMNPV gene encoding a 35,000-molecular-weight protein (35K protein) located on the *Eco*RI S genome fragment (Fig. 1A). Transcription of the predominant mRNA, α_1, for the gene encoding the 35K protein (hereafter referred to as the 35K gene) occurs within the first hour of infection, peaks early, and declines later (9, 10). The gene itself is flanked on the left by an early 94K gene and on the right by the *hr5* enhancer element (Fig. 1B). Both early genes are surrounded by late viral genes and, since they are divergently transcribed from a common intergenic region (Fig. 1C), may share common regulatory signals.

Our approach to identifying sequences important for 35K gene expression involved analysis of the effects of site-directed mutagenesis on the 35K gene promoter region fused to the chloramphenicol acetyltransferase gene (CAT). CAT has been used successfully as a sensitive reporter of promoter activity in insect cells (3, 5, 16). When linked in *cis* to the AcMNPV *hr5* enhancer and cotransfected with the *trans*-activating viral gene IE1, the 35K gene fragment behaved as an early viral promoter, since transcription was

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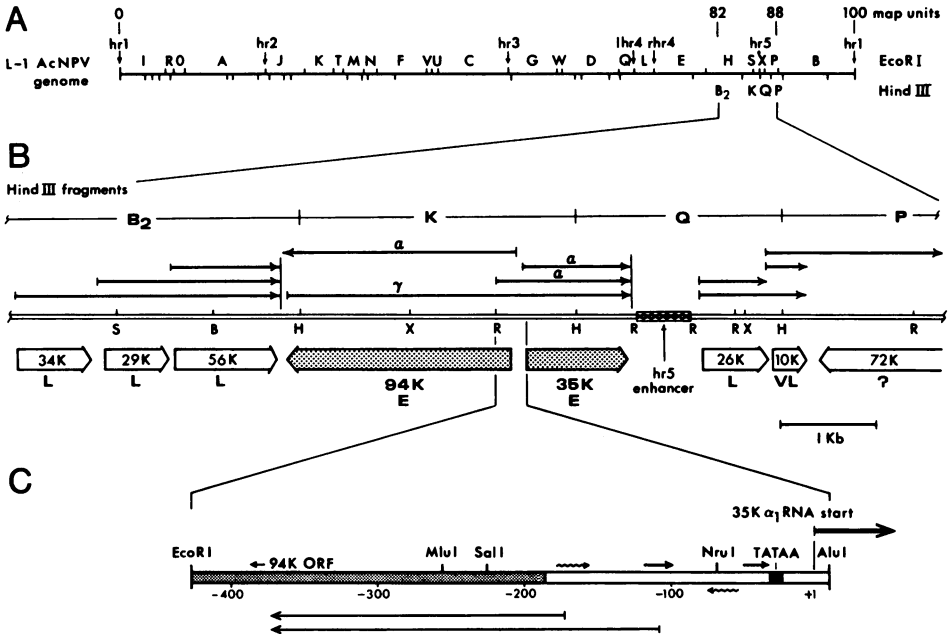

FIG. 1. Map position of the 35K gene encoded by the *Eco*RI-S genome fragment of AcMNPV. (A) Linear representation of the AcMNPV L-1 (L-1 AcNPV) genome showing *Eco*RI and *Hin*dIII restriction fragments and the positions of enhancer (*hr*) regions. (B) Position of the 35K gene-coding region relative to those of the other viral-coding regions (large open arrows) between 82 and 88 map units. The entire region has been sequenced and transcripts have been mapped (10, 17, 21, 23, 29, 31); predominant RNAs are shown at the top. Abbreviations: B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; S, *Sma*I; X, *Xho*I; E, early; L, late; VL, very late genes. (C) Transcriptional map of the 437-bp *Eco*RI-*Alu*I restriction fragment containing 35K gene promoter sequences. The start site (position +1) for the major 35K gene RNA, α_1, and the divergent 94K gene RNA start sites (bottom) are shown. Direct and inverted repeats are depicted by straight and wavy arrows, respectively. ORF, Open reading frame.

initiated exclusively from the early RNA start site. In contrast, when inserted at an alternate position within the genome of recombinant viruses (referred to as v35K-CAT recombinants), the promoter fragment used the early RNA start site during the early stage of infection but switched later to an alternate start site in a pattern identical to that of the wild-type 35K gene promoter. On the basis of 5′ deletion analyses, regulatory sequences for both the early and late promoter activities were identified.

MATERIALS AND METHODS

Virus and cells. AcMNPV strain L-1 was propagated in *Spodoptera frugiperda* IPLB-SF21 cells as described previously (9). Cell monolayers (10^7 cells per 100-mm-diameter plate) were inoculated with nonoccluded extracellular virus at a multiplicity of 20 PFU per cell. After a 1-h adsorption period, residual inoculum was removed, and the monolayers were washed and fed with TC100 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum. Time zero was defined as the point at which residual inoculum was removed.

Plasmids. Recombinant plasmid DNAs were constructed and propagated by standard procedures (24). The transient expression vector pCAT-*hr5* (Fig. 2A) was assembled by first subcloning the AcMNPV-derived polyadenylation sequence B_2H (84.4 to 85.1 map units) via insertion of a 880-bp *Bam*HI-*Hin*dIII fragment (repaired with the Klenow fragment at the *Hin*dIII end) into the *Bam*HI and *Sma*I sites of pUC18, respectively. The CAT gene, including its own initiator and terminator codons, was then excised from plasmid pRSVcat (13) as a 785-bp *Hin*dIII-*Bam*HI fragment and inserted at the *Bam*HI and *Hin*dIII sites of the B_2H plasmid. To accommodate various promoter-containing fragments, a polylinker was inserted at the *Hin*dIII site to produce plasmid pCAT. pCAT-*hr5* vectors were then constructed by adding a single copy of the AcMNPV *hr5* enhancer by inserting the 484-bp Klenow fragment end-repaired *Mlu*I DNA fragment, which has been shown previously to encompass *hr5* (17), at either the upstream *Nde*I site [*hr5*(Up)] or the downstream *Kpn*I site [*hr5*(Dwn)]; both pCAT restriction sites were repaired with T4 DNA polymerase prior to ligation. Opposite orientations of *hr5* were isolated: genome sense and antisense strands (+ and −, respectively). pCAT and pCAT-*hr5* control plasmids lacking promoter sequences were constructed by excising the polylinker between *Sma*I and *Hin*dIII sites followed by blunt-end ligation. Plasmid pIE1 was subcloned from the *Hin*dIII G genome fragment of AcMNPV L-1 (kindly provided by P. Safer, Genetics Institute, Cambridge, Mass.) by excising the 3.0-kbp *Cla*I-*Hin*dIII fragment and inserting it into the *Hin*dIII and *Acc*I sites of pUC19. pIE1 contains exon 1 of the IE1 gene with its own promoter but lacks exon 0, mapping 4 kbp upstream (6, 18).

Flanking 5′-noncoding sequences of the 35K gene located on a 437-bp *Eco*RI-*Alu*I fragment (Fig. 1C) were inserted into the various pCAT vectors, to give p35K-CAT reporter plasmids. Figure 2B illustrates the structure of one such plasmid, p35K–CAT-*hr5*(Up$^+$). The unidirectional *Exo*III deletion procedure (20) was used to generate 5′ deletions of the 35K gene promoter fragment, beginning at the upstream *Eco*RI site at position −426. Deletion endpoints were determined by nucleotide sequencing of plasmid DNA by using the universal primer-binding site adjacent to the pCAT-*hr5* polylinker (Fig. 2A).

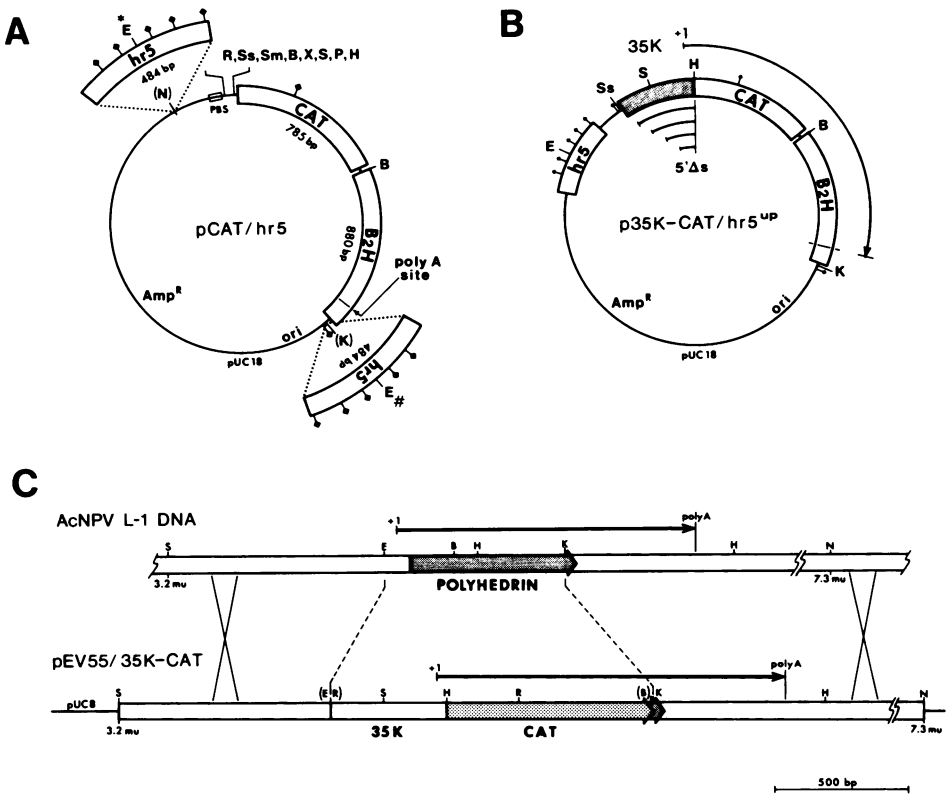

FIG. 2. (A) General structure of the transient expression vector pCAT-*hr5*. Each plasmid contains only a single copy of the AcMNPV *hr5* enhancer inserted (dotted line) either at the *Nde*I site (*) or at the *Kpn*I site (#) corresponding to plasmids pCAT-*hr5*(Up) and pCAT-*hr5*(Dwn), respectively. The genome sense orientation (+) of *hr5* is shown. (B) Structure of plasmid p35K–CAT-*hr5*(Up$^+$). The shaded region depicts inserted 35K gene promoter sequences. The major CAT-encoding RNA was initiated within the 35K gene promoter and extended through CAT, as shown. (C) Construction of v35K-CAT recombinant viruses by gene replacement. Recombination between wild-type AcMNPV DNA (top sequence) and the transplacement plasmid pEV55-35K–CAT (bottom sequence) resulted in replacement of the polyhedrin promoter and protein-coding sequences (shaded) with the chimeric 35K gene promoter-CAT sequences. Restriction site abbreviations: E, *Eco*RV; N, *Nde*I; Ss, *Sst*I; Sm, *Sma*I; B, *Bam*HI; X, *Xba*I; S, *Sal*I; P, *Pst*I; H, *Hin*dIII; K, *Kpn*I; R and diamond symbols, *Eco*RI. mu, Map units; PBS, universal primer-binding site. Parentheses denote restriction sites that were lost during cloning procedures.

Recombinant viruses. AcMNPV recombinants were constructed by standard gene replacement methods (27, 28). In each case, the wild-type AcMNPV polyhedrin gene was replaced with the CAT that was previously fused to 5′-flanking sequences of the 35K gene (Fig. 2C). Transplacement plasmids (pEV55–35K-CAT) were constructed by using the transplacement vector pEV55 (27, 28), which was kindly provided by D. W. Miller (Genetics Institute). In brief, promoter-CAT fusions were excised from plasmid p35K–CAT-*hr5* (or the indicated 5′ deletion plasmids pΔ35K–CAT-*hr5*) by digestion with *Sst*I, followed by end repair with T4 DNA polymerase, and *Bam*HI digestion. The resulting fragments were inserted into pEV55, which was previously cleaved with *Eco*RV (position −92 relative to the polyhedrin ATG initiator codon at position +1) and *Bgl*II of the polylinker. The resulting pEV55–35K-CAT plasmids (Fig. 2C) were cotransfected with wild-type AcMNPV DNA, and recombinant viruses exhibiting the occlusion-negative phenotype were isolated. Recombinants were further purified by two consecutive plaque isolations. Proper insertion of 35K-CAT sequences was confirmed by restriction mapping and Southern blot analysis of recombinant virus DNA (data not shown).

Transfections. *S. frugiperda* cell monolayers were transfected with plasmid or viral DNAs as described previously (M. D. Summers and G. E. Smith, Texas Agric. Exp. Station Bull. No. 1555, 1987). In brief, 1 to 10 μg of supercoiled DNA, which was purified twice on CsCl gradients, was added to 950 μl of HEBS (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.05]–0.14 M NaCl–5 mM KCl–0.7 mM Na_2HPO_4–6 mM glucose) and mixed gently with 50 μl of 2.5 M $CaCl_2$. After 30 min at room temperature, the DNA precipitate was added dropwise to cell monolayers that were overlaid previously with Grace's insect medium (GIBCO Laboratories) supplemented with 10% heat-inactivated fetal bovine serum. After a 4-h incubation at 27°C, the monolayers were washed twice with TC100 medium and were then fed TC100 plus 10% heat-inactivated fetal bovine serum.

CAT assays. Cells were harvested by scraping them 24 h after transfection. After they were washed twice with ice-cold phosphate-buffered saline, the cells were suspended in 0.25 M Tris (pH 7.8) and subjected to three freeze-thaw cycles. Cellular debris was removed by centrifugation at 16,000 × *g*, and the supernatants were stored at −80°C. Cells infected with recombinant viruses were treated in an identical manner. Cell lysates were assayed for CAT activity as described by Gorman et al. (14) by using 0.1 μCi of [^{14}C]chloramphenicol (54 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) per assay. Reactions were conducted at 37°C for 30 min. The acetylated forms of chloramphenicol were separated by thin-layer chromatography, located by autora-

diography, excised, and quantitated by liquid scintillation counting. To ensure the linearity of the assay, cell lysates were diluted such that less than 40% of the input chloramphenicol was acetylated.

S1 nuclease and primer extension analyses of RNA. Total RNA from cells transfected with plasmid DNA or infected with virus was extracted by using the guanidine isothiocyanate-cesium chloride method (5). S1 nuclease mapping of 5′ ends of RNA transcripts was conducted by using a modification of the procedure of Weaver and Weissman (34) and single-stranded DNA probes that were synthesized as described previously (2). To prepare the probes, an oligonucleotide primer (see below) labeled at the 5′ end with ^{32}P was annealed to the appropriate single-stranded M13mp DNA and extended with the Klenow fragment. The DNA product was cleaved with *Hin*cII (at the unique *Sal*I site), and the resulting 5′-end-labeled probe was purified by electrophoresis on a denaturing alkaline-agarose gel. The DNA probe for wild-type 35K gene RNAs was prepared by using the primer CGTCTGGGACACGTCG (positions +53 to +68 relative to the RNA start site at position +1) that was annealed to an M13mp19 clone of the 1.46-kbp *Eco*RI-S AcMNPV genome fragment. The DNA probe for 35K-CAT RNAs was prepared by using the primer CGGTGGTATATCCAG (positions +66 to +80 within the CAT-coding region) annealed to an M13mp19 clone of the 1.23-kbp *Sst*I-*Bam*HI fragment derived from plasmid p35K-CAT. Total RNA (50 μg) was hybridized at 30°C to both probes (50,000 cpm) in 75% formamide–20 mM Tris (pH 7.4)–0.4 M NaCl–1 mM EDTA–0.1% sodium dodecyl sulfate and was then treated with S1 nuclease as described previously (9).

Primer extension analysis of 35K-CAT RNA was conducted by using a 63-base primer extending clockwise from the *Hin*dIII site (positions +18 to +80) of p35K-CAT (Fig. 2B). The primer was prepared as described above for the CAT-specific S1 nuclease protection probe, except that the 15-base oligonucleotide primer was extended with the Klenow fragment in the presence of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham), cleaved with *Hin*dIII, and purified by denaturing polyacrylamide gel electrophoresis. The 63-base primer (50,000 cpm) was annealed overnight at 25°C to total RNA by using the hybridization conditions given above and was subsequently extended with 500 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.) in 50 mM Tris (pH 8.3)–75 mM KCl–3 mM $MgCl_2$–10 mM dithiothreitol–500 μM each of the four deoxynucleoside triphosphates–50 μg actinomycin D per ml–50 μg of bovine serum albumin per ml–1 mM vanadyl ribonucleoside complex. Primer extension products and S1 nuclease-resistant fragments were denatured and subjected to electrophoresis on 6% polyacrylamide–8 M urea–TBE (100 mM Tris borate [pH 8.3], 2 mM EDTA) gels, followed by autoradiography. The dideoxy chain termination method of Sanger et al. (33) was used to generate [α-^{35}S]ATP-labeled sequencing ladders with the same primer-template combination that was used to prepare the extension primer and the respective S1 nuclease protection probes.

RESULTS

Early and late RNA start sites for the 35K gene. To initiate studies designed to locate the 35K gene promoter, the precise 5′ end of the predominant mRNA for the 35K gene, α_1 (1.07 kb), was determined. Since previous primer extension analyses revealed no evidence for splicing at the 5′ end

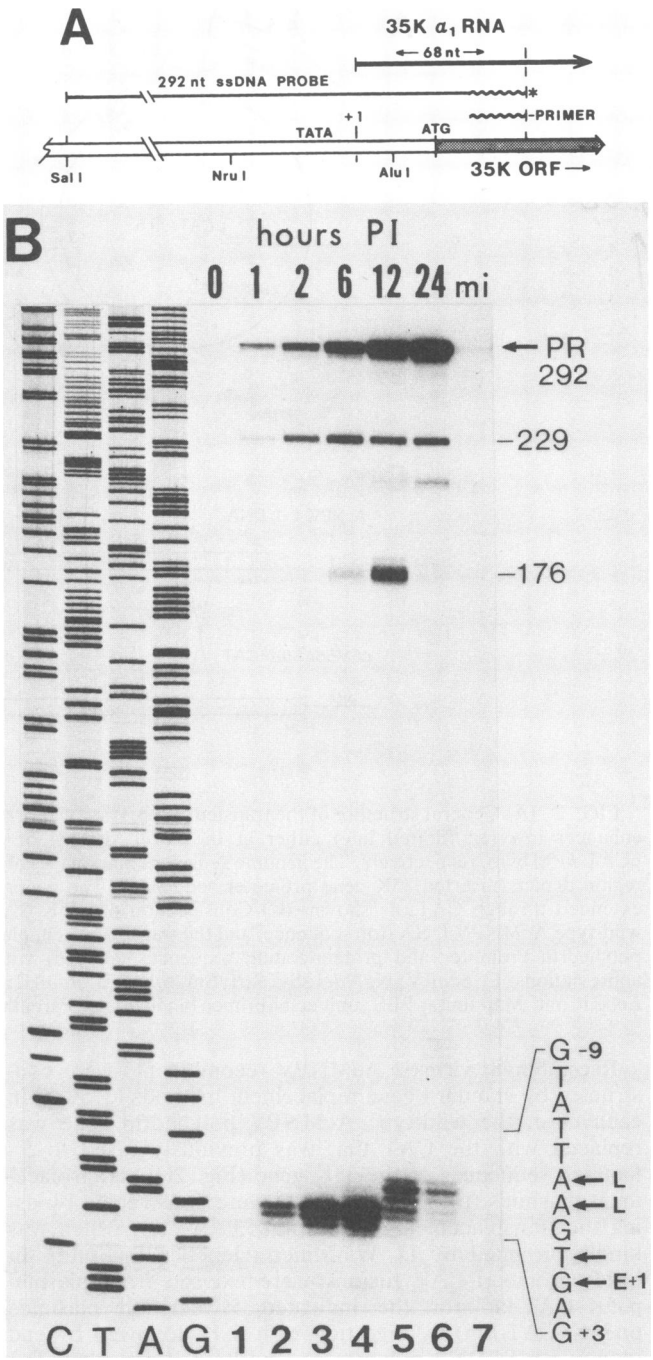

FIG. 3. High-resolution S1 nuclease mapping of the 5′ end of major RNA (α_1) of the 35K gene. Total RNA (50 μg) isolated from cells at the indicated times postinfection (PI) with wild-type AcMNPV was hybridized to the 292-base single-stranded DNA (ssDNA) probe (A); the asterisk denotes the position of the 5′ end label. ORF, Open reading frame; nt, nucleotide. After S1 nuclease treatment, the resistant fragments were subjected to polyacrylamide gel electrophoresis and autoradiography (B). Also included are the S1 nuclease-resistant fragments generated after hybridization to mock-infected (mi) RNA (lane 7) and the position of the full-length probe (PR). The 16-base primer used to prepare the single-stranded DNA probe (A) was also used to generate the sequencing ladder (B). Sizes (in bases) of S1 nuclease-protected fragments and the nucleotide sequence of the major initiation sites for early (E) and late (L) α_1 RNAs are indicated on the right.

TABLE 1. Effect of *hr5* enhancer and IE1 on p35K-CAT expression

Test plasmid	*hr5* orientation[a]	Relative CAT expression (pmol/min per 10^6 cells) of[b]:		Fold increase with:	
		Plasmid alone	Plasmid + IE1	*hr5*[c]	IE1[d]
p35K-CAT	None	0.2 ± 0.2	1,310 ± 200	1	
p35K-CAT-*hr5*	Up^+	14.6 ± 0.6	16,100 ± 3,500	12	1,100
p35K-CAT-*hr5*	Up^-	25.8 ± 0.3	21,500 ± 1,800	16	800
p35K-CAT-*hr5*	Dwn^+	11.4 ± 1.9	33,400 ± 500	26	3,000
p35K-CAT-*hr5*	Dwn^-	16.8 ± 3.8	26,800 ± 5,600	20	1,600
pCAT	None	1.0 ± 0.4	130 ± 30		131
pCAT-*hr5*	Dwn^+	0.2 ± 0.03	390 ± 20		1,900

[a] Abbreviations: up, Upstream; dwn, downstream, + and −, orientation.
[b] Transfections were conducted in triplicate by using 10 μg of test plasmid alone or 10 μg of test plasmid plus 1 μg of plasmid pIE1 per 2×10^6 cells.
[c] Fold increase was calculated from values of CAT activity in cells cotransfected with the indicated test plasmid and pIE1 and then normalized to p35K-CAT (lacking *hr5*).
[d] Fold increase was calculated from the ratio of CAT activity in cells cotransfected with pIE1 to that in cells without pIE1.

of the α_1 RNA (9), high-resolution S1 nuclease mapping was used. Total RNA, which was isolated from cells at various times after infection, was hybridized to a 5′-end-labeled DNA probe encompassing the α_1 initiation site and was then digested with S1 nuclease. Abundant α_1 transcripts, mapping predominately to G and T residues (hereafter designated as positions +1 and −1, respectively), were detected within the first hour after infection and increased through 2 and 6 h (Fig. 3). The early RNA start site was located 26 nucleotides upstream from the ATG initiation codon of the 35K gene. At late times (12 to 24 h), during the period of declining α_1 transcription, the RNA start site switched to a pair of A residues located several nucleotides farther upstream (positions −3 and −4, respectively), suggesting that different RNA initiation signals are used during the early and late periods of infection. In addition, longer S1 nuclease-resistant fragments of 176 and 229 bases were observed late in infection and represented minor viral RNAs with unknown functions that were mapped previously (9). The full-length probe (292 bases) was protected by longer α_2 and γ RNAs transcribed from positions upstream (Fig. 1) from the end of the DNA probe. No fragments were protected by RNA from mock-infected cells.

Transient expression of the 35K gene promoter. In order to define the functional promoter and potential regulatory signals relative to the RNA start sites mapped above, we first constructed chimeric reporter plasmids to moniter 35K gene promoter activity in transient expression assays. To this end, transient expression vectors (pCAT) were used that contained the protein-coding sequences of the CAT gene followed immediately by the AcMNPV polyadenylation sequence B_2H (Fig. 2A). B_2H contains polyadenylation signals for RNAs originating within the *Hin*dIII-B_2–*Eco*RI-H region (29) as well as RNAs transcribed in the opposite direction from the *Hin*dIII-K region (10). The 35K gene promoter, which was located on a 437-bp *Eco*RI-*Alu*I fragment (Fig. 1C), was inserted immediately upstream from CAT such that the resulting plasmids (p35K-CAT) contained sequences 426 bp upstream from the α_1 RNA start (position +1) and the first 12 of 26 nucleotides making up the 5′-noncoding RNA leader (Fig. 2B).

In transfected cells, the full-length, 437-bp promoter fragment alone directed only background levels of CAT activity (Table 1). However, addition of the *cis*-linked *hr5* enhancer resulted in a large increase (60- to 130-fold) in CAT expression; this increase was observed when *hr5* was positioned immediately adjacent to the promoter fragment (upstream) or adjacent to the B_2H poly(A) sequence (downstream) and in either orientation. *hr5* had no effect on CAT expression from the control pCAT-hr5 lacking the promoter fragment. Cotransfection of each p35K-CAT plasmid with plasmid pIE1 containing the *trans*-activating IE1 gene (18) from AcMNPV L-1 produced an even greater increase in CAT expression (Table 1). This stimulation did not require *cis* linkage of the test plasmid to *hr5*; however, the presence of *hr5* yielded a 12- to 26-fold increase above that provided by pIE1 alone. Thus, cotransfection with pIE1 resulted in an 800- to 3,000-fold increase in CAT activity from the target plasmids containing the 35K gene promoter and *hr5* (p35K–CAT-*hr5*). Interestingly, the degree to which expression from the promoterless pCAT-*hr5* control plasmid was stimulated by pIE1 was similar to that of the promoter-containing pCAT-*hr5* plasmids, although the overall level of CAT activity was low in comparison.

5′ Deletion mutagenesis of the 35K gene promoter. To delineate the 5′ boundary of sequences that are important for promoter activity, unidirectional *Exo*III deletions of the 437-bp promoter fragment were constructed and inserted into vector pCAT-*hr5*(Up), generating a series of plasmids, pΔ35K–CAT-*hr5*(Up), that contained progressively smaller promoter fragments from positions −426 to −4 (Fig. 4C). The 5′ to 3′ deletion plasmids were assayed for CAT expression after cotransfection with pIE1 (Fig. 4A). Each plasmid was transfected in triplicate for an individual experiment, and the average values are shown in Fig. 4; the actual CAT activities varied less than twofold between experiments, but they were internally consistent when activity from deletion plasmids was normalized to that of the full-length promoter plasmids. Deletion of nucleotides to position −322 exhibited no effect on promoter activity. However, deletions from positions −322 to −55 reduced activity by about 40%; and further deletions from positions −55 to −23, which included the putative TATA box, reduced overall activity by 80%. Unexpectedly, deletion of sequences to within four nucleotides (position −4) of the early start site exhibited significant levels of CAT expression relative to that of control pCAT-*hr5* lacking a promoter. To test whether this effect was due to the position of the *hr5* enhancer relative to the RNA start site, the same 5′-deleted promoter fragments were inserted into vector pCAT-*hr5* (Dwn), thereby positioning *hr5* at a constant distance downstream from the RNA start site. In this series of plasmids, progressive deletions resulted in a more uniform reduction of CAT expression (Fig. 4B). Moreover, deletion to position −31 and below reduced activity to near background levels. Despite their differences in overall CAT expression because

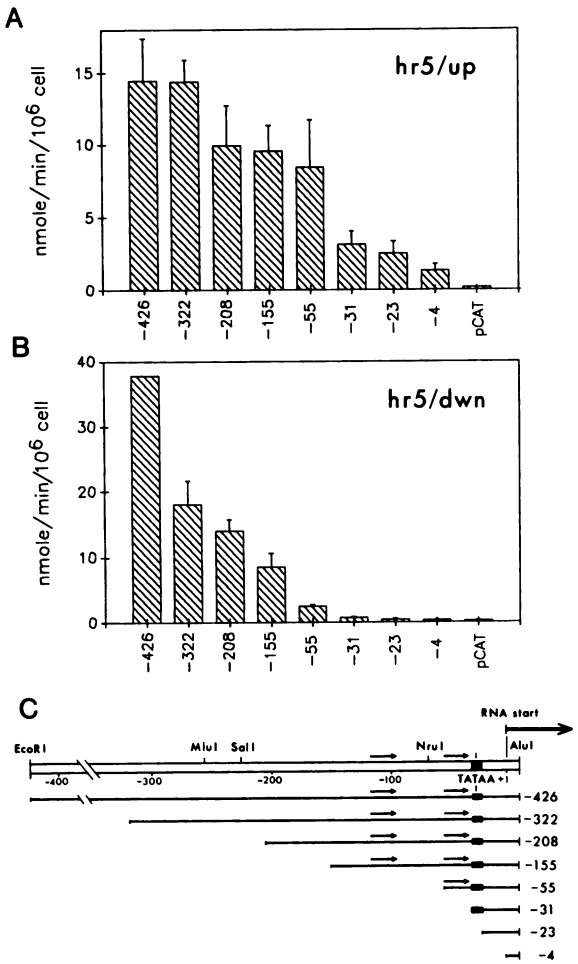

FIG. 4. Effect of 5′ deletion mutagenesis on 35K gene promoter activity assayed by transient expression. Cells were cotransfected with plasmid pIE1 (1 μg) and p35K–CAT-*hr5* plasmids (10 μg) containing the full-length promoter fragment (position −426 relative to the RNA start site at position +1) or fragments deleted from the 5′ end to the indicated nucleotide and were then assayed 24 h later for CAT. CAT expression from 5′ deletion plasmids pΔ35K–CAT-*hr5*(Up) (A) and pΔ35K–CAT-*hr5*(Dwn) (B) containing the *hr5* enhancer at upstream (up) or downstream (dwn) positions, respectively, is shown. CAT expression from the negative control, plasmid pCAT-*hr5*, is indicated in panels A and B. Transfections were conducted in triplicate; standard deviations are indicated by the error bar above each averaged value. (C) Location of the various 5′ deletions of the 35K gene promoter fragment relative to the locations of the major RNA start site and early consensus repeats (small arrows).

of the position of the enhancer, both series of deletion plasmids displayed the greatest proportional change in CAT activity on removal of sequences between positions −155 and −31.

The effect of 5′ to 3′ deletions on the site of initiation and the relative levels of transcription from the 35K gene promoter in transient expression assays was also determined. Primer extension mapping of RNA from transfected cells (Fig. 5) demonstrated that CAT-specific RNA from plasmids p35K–CAT-*hr5*(Up) and p35K–CAT-*hr5*(Dwn) was initiated from the same site (position +1) that was used early in infection by the 35K gene promoter in its normal position within the viral genome. The presence of one extension product (81 nucleotides long) indicated that a single early initiation site was used (Fig. 6) and further suggested that the heterogeneous bands encompassing position +1 in S1 nuclease analyses (Fig. 3; see Fig. 8) represented staggered ends generated by nuclease digestion. For both series of 5′ deletions, pΔ35K–CAT-*hr5*(Up) and pΔ35K–CAT-*hr5* (Dwn), the amount of RNA from position +1 paralleled the level of CAT activity observed. For example, in the *hr5*(Up) series, deletion to position −31 significantly reduced transcription, while deletion to positions −23 and −4 reduced transcription below the limits of detection. In addition, CAT-related RNAs were initiated from within pUC19 vector sequences from a position 50 bases downstream (clockwise) from the *hr5* enhancer of the *hr5*(Up) plasmids (Fig. 5B, arrows). The relatively constant levels of these RNAs, including those for promoter deletions at positions −23 and −4 that exhibited greatly reduced levels of CAT activity (Fig. 4A), and their unusually long 5′-noncoding leaders (from 650 bases for the full-length promoter plasmid to 230 bases for the shortest deletion plasmid) suggested that these RNAs contributed little, if any, to the total CAT activities of the p35K–CAT-*hr5*(Up) plasmids. We did not rule out the possibility that these vector RNAs contributed a proportionally higher level of CAT activity for the smallest deletion plasmids at positions −23 and −4. These same RNAs were not detected in cells transfected with pΔ35K–CAT-*hr5*(Dwn) plasmids (Fig. 5C), indicating that all CAT activity was derived from the 35K gene promoter insert. Transcription of the *hr5*(Up) vector RNAs may therefore be an effect of the enhancer on a normally silent vector promoter located nearby.

Sequences required for early viral transcription. To determine whether the 35K gene promoter behaved in a similar manner when inserted within the viral genome, a setting that more closely resembles presentation of viral DNA within the host nucleus, we next examined the temporal expression of CAT from recombinant v35K-CAT viruses. These studies permitted the identification of promoter sequences that mediate early viral expression, which is not possible with transient assays. Standard gene replacement procedures were used to construct recombinant viruses in which the full-length promoter fragment or 5′ deletions thereof were fused to CAT and inserted into the AcMNPV genome at the site of the nonessential polyhedrin gene (3.1 to 3.6 map units). In each viral recombinant polyhedrin sequences, extending from the *Eco*RV site (position −92 relative to the ATG initiator codon at position +1) to the downstream *Kpn*I site (position +683), were replaced with 35K-CAT sequences. This removed the transcriptional control sequences (excluding distal enhancers) and 90% of the coding sequences for the polyhedrin gene (25, 30); CAT-specific transcripts were therefore initiated from the 35K gene promoter but terminated at the normal polyhedrin site (Fig. 2C).

The effects of 5′ deletions on overall promoter activity and temporal expression were determined by measuring CAT activity in cells at various times after infection. Cells infected with v35K-CAT recombinants containing the full-length promoter (position −426) exhibited early expression of CAT that paralleled the early transcription of the 35K gene in wild-type virus-infected cells. CAT activity was first detected 2 h after infection and continued to accumulate exponentially through 26 h (Fig. 7A). Deletion of nucleotides above position −155 had no apparent effect on the levels or timing of CAT expression, as demonstrated by recombinants Δ208 and Δ155. Recombinant Δ55, on the other hand, synthesized only background levels of CAT activity early in

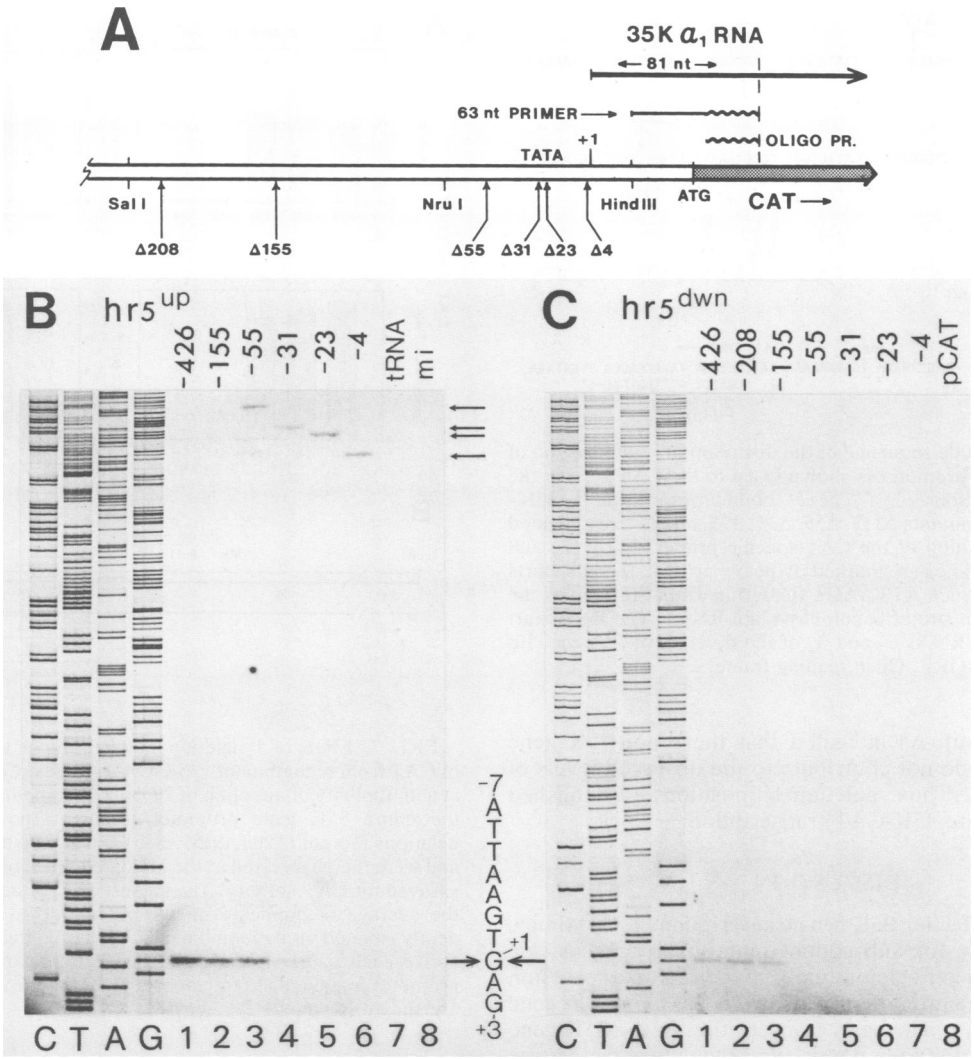

FIG. 5. Primer extension analysis of the 5′ ends of 35K-CAT RNAs from transfected cells. Total RNA (12.5 μg) isolated from 10^7 cells 24 h after cotransfection with 1 μg of pIE1 and 10 μg of deletion plasmids pΔ35K–CAT-*hr5*(Up) (B) or pΔ35K–CAT-*hr5*(Dwn) (C), containing the full-length promoter fragment (position −426) or 5′ deletions to the indicated nucleotide (positions −208, −155, −55, −31, −23, and −4), was hybridized to the 63-nucleotide (nt) primer depicted in panel A. After extension with reverse transcriptase, the products were analyzed by electrophoresis and autoradiography (B and C). A 15-base oligonucleotide primer (OLIGO PR.) (A) was used to generate the sequencing ladders, and the radiolabeled 63-nucleotide primer that was used for extension. No extension products were generated after hybridization to tRNA (B, lane 7), RNA from mock-infected (mi) cells (B, lane 8), or RNA from cells transfected with control plasmid pCAT-hr5(Up) (pCAT) (C, lane 8). The autoradiogram in panel B was exposed twice as long as that in panel C, to better illustrate minor bands.

infection (0 to 6 h) but exponentially increasing levels later (12 to 26 h); moreover, Δ55 CAT activity at 26 h was comparable to that provided by the full-length promoter (Fig. 7A). Thus, the approximate 5′ boundary of sequences important for early, but not late, promoter activity was located about 155 bp upstream from the α_1 RNA start site (Fig. 6). Recombinant Δ4 was defective for CAT expression both early and late in infection.

Both high-resolution S1 nuclease mapping and primer extension analyses indicated that 35K-CAT RNA was initiated from the full-length promoter (position −426) in a manner identical to that of the wild-type promoter (Fig. 8). Early in infection (6 h) the only CAT-specific RNA that was detected was initiated from the +1 position. At later times (24 h) initiation of the predominant RNAs shifted upstream to the late start sites at positions −3 and −4; multiple initiation sites mapping further upstream were also observed. As demonstrated by recombinant Δ55, deletion of sequences to position −55 dramatically reduced use of the early start site but did not affect that of the late start sites. Prolonged autoradiographic exposures (data not shown) revealed only minor usage of the early start site at 6 h. Thus, the observed levels of early and late 35K-CAT RNAs (Fig. 8) were consistent with the early and late expression of CAT by Δ55 (Fig. 7). Additional S1 nuclease-protected fragments were detected that mapped to the junction of the inserted 35K gene promoter fragment and upstream viral sequences of recombinant Δ55 (Fig. 8B, arrow). The absence of the same fragments after primer extension (Fig. 8C) demonstrated that they represented RNAs originating within the upstream polyhedrin-related sequences. Such RNAs also accounted for similar levels of S1 nuclease-protected fragments at positions −3 and −4 of recombinant Δ4 (Fig. 8B). However, the absence of CAT activity both early and late

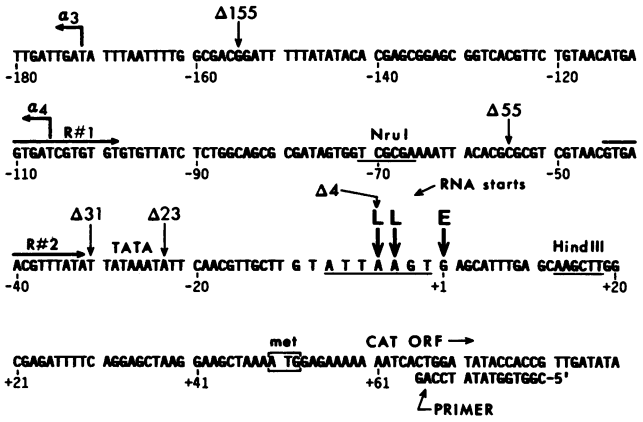

FIG. 6. Nucleotide sequence of the upstream promoter region of the 35K gene. The promoter is shown fused to the CAT gene at the *Hin*dIII site (position +15). The 5′ boundaries of the pΔ35K–CAT-*hr*5 deletion mutants Δ155, Δ55, Δ31, Δ23, and Δ4 are indicated along with the position of the CAT-specific primer. Early (E) and late (L) RNA start sites are marked by heavy arrows; the late starts lie within the sequence ATTAAGT (underlined) that resembles the consensus initiation site of baculovirus late RNAs. The RNA start sites for the major RNAs α_3 and α_4 of the divergent 94K gene (10) are also indicated. ORF, Open reading frame.

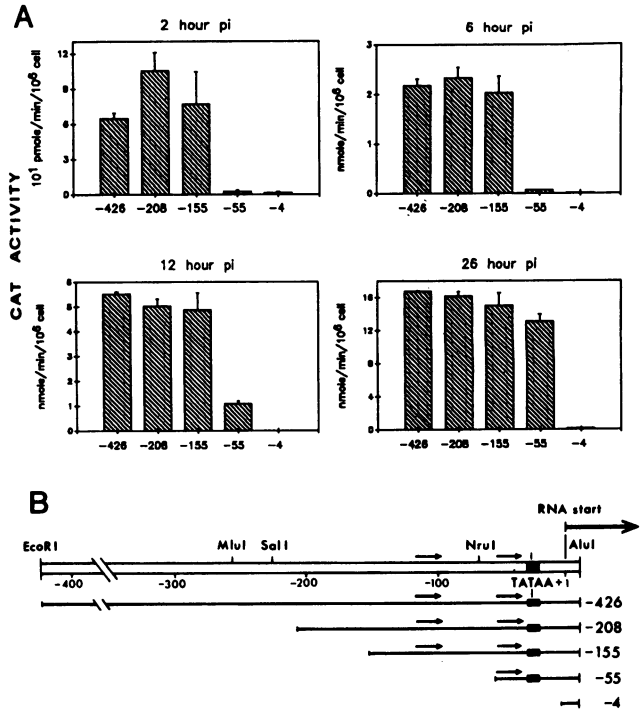

FIG. 7. Effect of 5′ deletion mutagenesis on temporal expression of CAT from recombinant v35K-CAT viruses. Cells were inoculated at a multiplicity of infection of 20, with recombinant viruses carrying the entire 35K gene promoter fragment (position −426) or 5′ deletions thereof (Δ208, Δ155, Δ55, and Δ4) fused to the CAT gene, and were then harvested at the indicated times postinfection (pi) and assayed for CAT activity. The values for CAT activity (A) represent the average of duplicate infections conducted with two independently isolated viral recombinants; standard deviations are indicated above each value. Location of the 5′ boundary of the full-length promoter fragment and fragments deleted to the indicated nucleotide are shown in panel B.

after infection with Δ4 indicated that these non-35K gene promoter RNAs do not contribute to the observed levels of CAT expression. Thus, deletion to position −4 abolished both early and late 35K-CAT transcription.

DISCUSSION

Viral factors affecting 35K gene transcription. To maximize promoter activity for subsequent mutational analysis, we first examined the effects of auxiliary factors on expression from the 35K gene promoter by using CAT as a reporter gene in transient assays. The early transcription of the 35K gene (Fig. 3) suggests that few, if any, viral regulatory factors are required for its expression. *cis* linkage of the AcMNPV *hr*5 enhancer, which is located immediately downstream from the C terminus of the 35K gene within the wild-type viral genome (Fig. 1), produced a significant increase (60- to 130-fold) in promoter activity since the promoter fragment alone was not active (Table 1). The stimulatory effect of *hr*5 did not require the presence of any viral protein. This contrasted with *hr*5-mediated enhancement of the delayed early 39K gene of AcMNPV that required the viral *trans*-activating regulator IE1 (16, 17). While not dependent on IE1, expression from the 35K-CAT plasmids, with or without the *hr*5 enhancer, was dramatically stimulated when IE1 was cotransfected as a cloned fragment, confirming that IE1 plays a major role in early gene *trans*-activation in transient expression assays (18). It has been shown previously (19) that pUC-derived plasmids linked to *hr*5 fail to replicate in *S. frugiperda* cells that are cotransfected with IE1. Thus, *hr*5-IE1 enhancement in transient assays is not the result of an amplification of DNA template but involves a stimulation of the rate of transcription, as suggested by nuclear run-on assays (17).

Whether the *hr* enhancer is required for early transcription of the 35K gene while it is embedded in the viral genome is not clear. Enhancers mediate the transcription of early genes of several small DNA viruses, including those of simian virus 40 and polyomavirus (for a review, see reference 15), and have been mapped near immediate-early genes of large DNA viruses, including those of herpes simplex virus type 1 and cytomegalovirus (1, 22). There was no obvious requirement, however, for a proximal *hr* enhancer for early expression of chimeric 35K gene promoter-CAT sequences in recombinant viruses (Fig. 7 and 8); the nearest viral enhancer (*hr*1) was positioned 5 kbp upstream from the site of gene insertion. Not ruled out was the possibility that the *hr* enhancer exerts an effect over distances greater than 5 kbp or that an as yet unidentified viral enhancer nearby was involved.

Our data suggest that regulation of the 35K gene promoter is analogous to that of the immediate-early genes α27 and α22/47 of herpes simplex virus type 1. These genes do not require de novo viral protein synthesis for expression, yet they are responsive to other immediate-early genes, including α4 and α0, that function as *trans*-activating regulators (12). Similarly, our results indicated that while IE1 plays a major role in the stimulation of 35K gene transcription in transient expression assays, the 35K gene promoter linked to *hr*5 did not require IE1. This is in contrast to results of an earlier study in which S1 nuclease protection assays were used to detect 35K gene-specific RNAs in transfected cells (18). We attribute the apparent discrepancy to an increased sensitivity provided by the use of CAT as a reporter of promoter activity. Genetic analyses will be required to

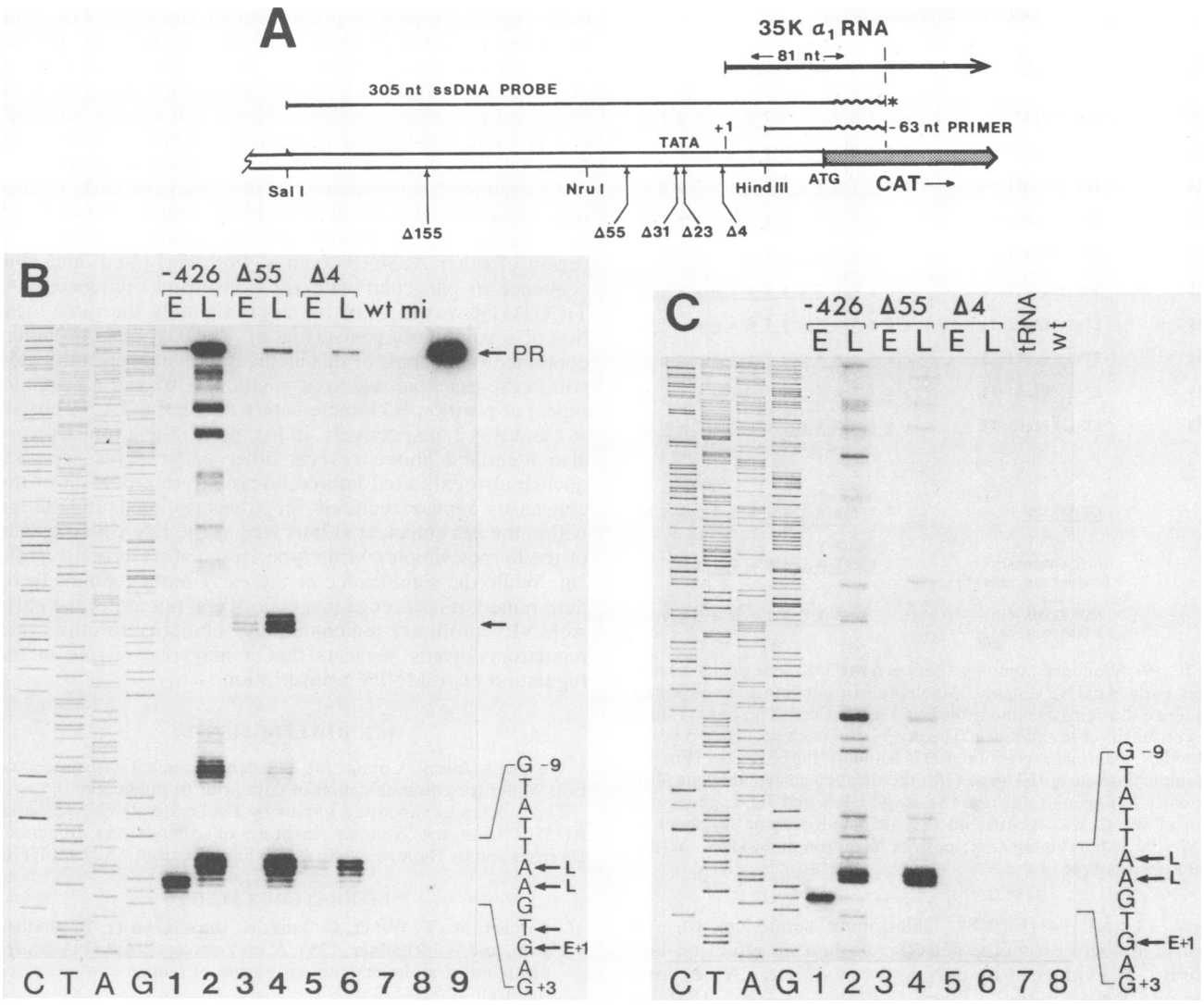

FIG. 8. S1 nuclease mapping and primer extension analysis of the 5′ ends of 35K-CAT RNAs from recombinant virus-infected cells. Cells were inoculated at a multiplicity of infection of 20, with the indicated recombinant v35K-CAT viruses carrying the full-length 35K gene promoter fragment (position −426) or the 5′ deletion mutants Δ55 and Δ4, respectively. Total RNA (30 μg) that was isolated 6 h (early [E]) and 24 h (late [L]) after infection was hybridized to the 5′-end-labeled, 305-base probe or the radiolabeled 63-base primer depicted in panel A. S1 nuclease-resistant fragments (B) and primer extension products (C) were analyzed by electrophoresis and autoradiography. No S1 nuclease-resistant fragments or primer extension products were generated after hybridization to RNA from wild-type (wt) AcMNPV-infected cells (24 h after infection), mock-infected (mi) cells, or tRNA. Abbreviations: ssDNA, Single-stranded DNA; nt, nucleotide; PR, full-length probe.

clarify the exact role of IE1 in stimulating viral transcription, including that of the 35K gene, in a normal infection.

Sequences required for 35K gene promoter activity in transient expression assays. When linked to *hr5* and cotransfected with pIE1, the full-length promoter fragment extending from positions −426 to +14 directed transcription from the early RNA start site at position +1 (Fig. 5), and therefore exhibited early promoter activity. The 5′ deletion mutagenesis of the promoter fragment resulted in a gradual reduction in CAT expression (Fig. 4) that paralleled transcription from position +1 (Fig. 5) and suggests that a large region is involved in regulating the overall level of 35K gene promoter activity in transient expression assays. That the effect of identical 5′ deletions differed according to the position of the *hr5* enhancer, either upstream or downstream relative to the RNA start site, also suggests that the enhancer exerts a position-dependent influence over the 35K gene promoter. Nevertheless, the greatest reduction in CAT expression and 35K-CAT transcription in transient assays was observed when sequences between positions −155 and −31 were deleted, suggesting that the 5′ boundary of the more influential elements is located about 155 nucleotides upstream from the early RNA start site.

Sequences required for early versus late viral transcription. In contrast to transient expression assays, 35K gene promoter sequences between positions −426 and −155 had no effect on the level of expression, early or late, of the 35K-CAT gene while they were embedded in the genome of recombinant viruses. In a pattern identical to that of the wild-type 35K gene, the full-length promoter directed synthesis of early transcripts from position +1 which were subsequently replaced with late transcripts starting upstream from posi-

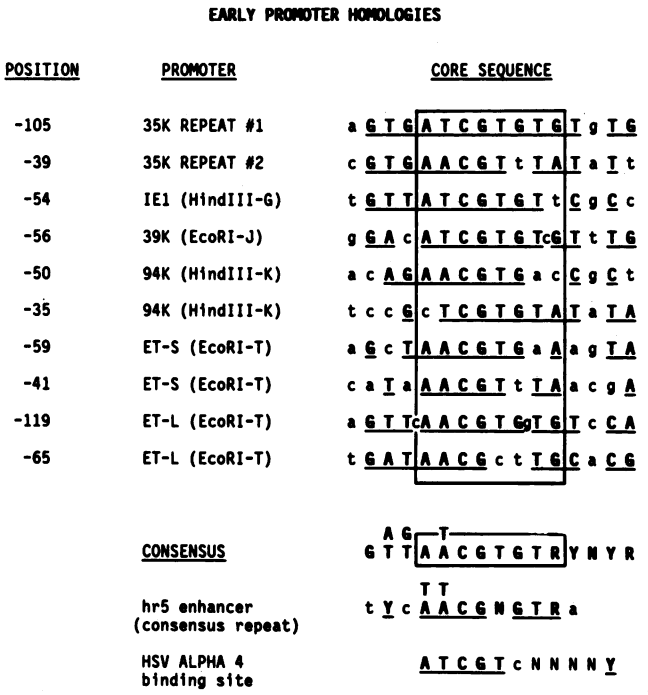

FIG. 9. Sequence homology between the 35K gene promoter and other early AcMNPV genes. Nucleotides matching the consensus sequence (bottom) are underlined and capitalized. The box outlines the consensus core sequence. The position of each sequence relative to the RNA start site (position +1) is listed for the 35K gene (Fig. 6), the immediate early IE1 gene (18), the delayed early 39K gene (16), the early 94K gene (10), and the early ET-S and ET-L genes (7), each of which was located on the indicated genome fragment of AcMNPV. Abbreviations: R, purine; Y, pyrimidine; HSV, herpes simplex virus type 1.

tions −3 and −4 (Fig. 8). Deletion of sequences to −55 abolished early promoter activity yet had no effect on late activity, as evidenced by normal levels of late CAT expression and RNAs initiated from positions −3 and −4 late in infection. These data, in addition to the observed temporal switch in the utilization of early versus late RNA start sites, suggest that the 35K gene promoter contains both early and late regulatory components. It is relevant in this regard to note that the sequence surrounding the RNA initiation sites for the 35K gene bears partial homology with the highly conserved sequence (A/T) (A/T)ATÅÅGNA(A/T) (the 35K gene sequence matches are underlined) located at the start site of late AcMNPV genes. This late gene motif constitutes the major determinant of transcription of the late polyhedrin gene (25, 30, 31a). Since the 35K gene late RNA start sites coincided with the most frequently used start sites (see asterisks in the sequence given above) within the conserved late motif, it is likely that the partially homologous sequence functions as the late regulatory component of the 35K gene promoter. Consistent with this hypothesis was the observation that deletion of part of the homolog (to position −4) dramatically reduced late CAT expression (Fig. 7).

Deletion mutagenesis also indicated that the 5′ boundary of elements potentiating early 35K gene transcription is located near position −155. Since minor levels of correctly initiated early RNAs were detected with recombinant v35K-CAT Δ55, the sequences between positions −155 and −55 function to regulate the overall level of early transcription. In its usual genomic position within the *Eco*RI-S region, the early regulatory signals predominated since the 35K gene promoter primarily directed transcription of early RNAs; only during declining 35K gene transcription did the late starts appear (Fig. 3). It is not clear why late 35K-CAT transcription was enhanced in recombinant viruses, although it is possible that the late regulatory component is sensitive to *cis*-related effects of position within the viral genome.

To examine the uniqueness of the 35K gene early regulatory sequences, the region between positions −155 and −55 was compared by computer with the upstream, noncoding region of other AcMNPV genes, both early and late. One sequence in particular with the consensus sequence A(A/T)CGTGTR was identified at approximately the same location of several early genes (Fig. 9). The 35K gene promoter contained two copies of this element: a distal repeat located within the essential region at position −105 and a proximal repeat at position −39 immediately above the TATA box (R # 1 and R # 2, respectively, in Fig. 6). Multiple repeats were also identified above several other early genes. The sequence also exhibited limited homology to a portion of the consensus repeat required for transcriptional stimulation within the *hr5* enhancer (17) as well as the DNA-binding site of the herpes simplex virus type 1 regulatory protein α4 (8, 26). While the significance of the early motif remains to be determined, its presence in a majority, if not all, of the early AcMNPV promoter regions and its similarity to other viral regulatory signals suggests that it may play a role in the regulation of AcMNPV transcription.

ACKNOWLEDGMENTS

We thank Joseph Corsini for excellent technical assistance and Lois Miller for communication of data prior to publication.

This work was supported in part by Public Health Service grant AI25557 from the National Institute of Allergy and Infectious Diseases and by Biomedical Research Support grant 2 S07 RR07170.

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