Identification of Upstream Promoter Elements Mediating Early Transcription from the 35,000-Molecular-Weight Protein Gene of *Autographa californica* Nuclear Polyhedrosis Virus

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Site-directed mutagenesis was used to examine the organization of *cis*-acting regulatory elements that comprise the promoter of the early 35,000-molecular-weight protein gene (35K protein gene) encoded by the *EcoRI*-S region of the baculovirus *Autographa californica* nuclear polyhedrosis virus. The promoter fragment, extending from positions –226 to +12 relative to the early RNA start site (position +1), was fused to the reporter gene encoding chloramphenicol acetyltransferase (CAT) and then inserted into the genome of recombinant viruses (3.96 map units) in order to ascertain the role of regulatory elements in the context of a normal infection. A combination of deletions and linker insertions revealed that early transcription was mediated by a basal (minimum) promoter, consisting of the TATA element (positions –30 to –25), that was in turn responsive to an upstream activating region located between –90 and –30. The TATA element exerted the single greatest influence on the level of early promoter activity and contained all information necessary to direct transcription from a site located 30 nucleotides downstream. The upstream activating region provided a 10- to 15-fold stimulation of transcription from the early +1 start site that was mediated by distinct DNA elements. These regulatory elements included two GC motifs (centered at positions –81 and –54, respectively), composed of alternating G and C residues, and a CGT motif (position –40) that contained the core sequence A(A/T)CGT(G/T). Each motif was required for full promoter activity during the early phase of infection. This organization that employs diverse *cis*-acting stimulatory elements is typical of promoters responsive to RNA polymerase II and may facilitate increased expression of *A. californica* nuclear polyhedrosis virus genes early in infection when the level of viral DNA for transcription is critically low.

Multiplication of *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV), the prototype of the polyhedrosis virus subgroup of the family *Baculoviridae*, is characterized by sequential and coordinately regulated stages of gene expression that result in the production of two morphologically and temporally distinct forms of infectious progeny. Expression of the early, late, and very late (occlusion-specific) classes of viral genes is regulated primarily at the level of transcription of the circular, ~128-kbp DNA genome (for reviews, see references 3 and 7). The molecular mechanisms by which AcMNPV discriminates between early and late classes of genes, and thereby executes its transcriptional program, is of considerable interest. Part of this control may be mediated by an unusual RNA polymerase switching mechanism that shifts from dependence on the host RNA polymerase II for early viral transcription to that of a virus-induced, α -amanitin-resistant RNA polymerase for late gene transcription (9, 18). Early and late phases of AcMNPV gene expression are also distinguished by a contrasting dependence on viral DNA replication; early viral transcription precedes the onset of DNA synthesis (approximately 6 h after infection) and, unlike late and very late transcription, is generally unaffected by metabolic drugs or viral mutations that inhibit DNA replication (reviewed in reference 7). As demonstrated by transient expression assays, at least one early AcMNPV gene (IE1) functions to

stimulate transcription of other viral genes (14). This suggests that AcMNPV employs various *trans*-activating factors (either virus encoded, host encoded, or both) to potentiate transcription and accomplish temporal control. It is expected that the specificity of this control is mediated by the interaction of such factors with the *cis*-acting regulatory elements comprising the promoter of each gene as well as nearby transcriptional enhancers.

As a first step in elucidating the mechanisms by which AcMNPV discriminates between early and late genes, we have examined the structure and organization of *cis*-acting DNA elements that direct transcription of the 35,000-molecular-weight protein gene (35K protein gene) located within the *EcoRI*-S region (86.8 to 87.8 map units [m.u.]) of the AcMNPV genome (Fig. 1A). This gene provides a useful model for studies on transcriptional regulation, since its promoter (35K promoter) is highly active and possesses both early and late control elements. During infection, the early 35K promoter element directs synthesis of an abundant, nonspliced mRNA, α_1 (Fig. 1B), that appears within the first hour after infection, peaks between 4 and 8 h, and declines thereafter (8, 17, 29). Later in infection, less abundant transcription is initiated from a sequence motif within the 35K promoter region, located near the early RNA initiation site, that is similar to the major determinant for late and very late AcMNPV promoters (33, 37, 38). The early 35K promoter is responsive to the transcriptional activator IE1 as well as the *cis*-acting AcMNPV enhancer, *hr5* (14, 29), that is positioned immediately downstream of the 35K protein gene within the viral genome (Fig. 1B). The upstream

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FIG. 1. Location and transcription of the AcMNPV 35K protein gene. (A) Linear map of the AcMNPV genome. The 35K protein gene is encoded by the *EcoRI* S genome fragment (86.8 to 87.8 m.u.) of the wild-type (wt) AcMNPV genome. The *EcoRI* genome fragments surrounding the 35K protein gene and the polyhedrin gene are indicated along with the positions of the AcMNPV transcriptional enhancers (*hr1* to *-5*). A site (3.96 m.u.) located immediately upstream from the polyhedrin gene was used for the insertion of CAT reporter sequences into the genome of recombinant 35K-CAT viruses. (B) Transcription map of the region encoding the 35K protein gene. An enlargement of the *EcoRI*-H and -S region (85.1 to 88.5 m.u.) depicts the 35K protein gene positioned between the divergent 94K protein gene on the left and the *hr5* enhancer (striped box) on the right. The 1.1-kb α_1 mRNA for the 35K protein gene and the α_3 and α_4 mRNAs for the 94K protein gene initiate from within the intergenic region. The 35K promoter fragment used in these studies extended from the *SalI* to *AluI* restriction sites (positions -226 to +12); the α_1 RNA start site is defined as position +1. Restriction site abbreviations: A, *AluI*; H, *HindIII*; M, *MluI*; N, *NruI*; S, *SalI*.

regulatory region of the 35K protein gene is shared by that of the 94K protein gene (encoding a 94,000-mol-weight protein) that is also transcribed early in infection but in the opposite direction.

In this report, we have identified sequence motifs that constitute both the promoter and the associated activating elements for early transcription of the 35K protein gene. Having previously determined that the region from 150 bp upstream to 12 bp downstream of the RNA start site was necessary for directing maximum levels of early transcription of the 35K protein gene (29), we fused this region to the reporter gene encoding chloramphenicol acetyltransferase (CAT). Mutations of the promoter fragment that produced alterations in the level of CAT expression were then used to define individual regulatory elements mediating early promoter activity. Recombinant viruses carrying the CAT gene under control of the 35K promoter were used so that the mutational effects on promoter activity could be assessed while embedded within the viral genome, a context that closely reproduced regulation of the 35K protein gene during the course of a normal infection. Our findings demonstrated that the TATA box was essential for early transcription and constituted the early basal (minimum) promoter for the 35K protein gene. Distinct DNA elements, located upstream from the TATA element, were involved in the stimulation of basal transcription. None of these elements affected transcription from the weaker late promoter, suggesting that the control elements for early baculovirus promoters differ substantially from those for late promoters.

MATERIALS AND METHODS

Cells and infection. *Spodoptera frugiperda* cell line IPLB-Sf21 (45) was propagated in TC100 growth medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2.6 mg of tryptose broth per ml and 10% heat-inactivated fetal bovine serum. For infection, cell monolayers were inoculated with extracellular virus from the indicated AcMNPV (L-1 strain) recombinants at a multiplicity of 20 PFU per cell. Residual inoculum was removed after a 1-h adsorption period. The cells were then washed, covered with growth medium, and incubated at 27°C. Time zero in the infection cycle designates the point at which the inoculum was removed.

Recombinant plasmids and site-directed mutagenesis. A series of plasmids that contained specific deletions of the 35K promoter fragment fused to the CAT gene were constructed as outlined in Fig. 2A. These plasmids, designated p($\Delta 3'$ -X/ $\Delta 5'$ -Y)35K-CAT, where X and Y denote the positions of the 3' and 5' deletions, respectively, relative to the RNA start site (position +1), were generated by using the unidirectional exonuclease III method (15), followed by the addition of *Bgl*III linkers (5'-CAGATCTG-3'). Plasmid p35K-CAT/*hr5* (29) was first used to generate deletion series p($\Delta 5'$ -Y)35K-CAT, in which sequences were progressively removed from the 5' side of the 35K promoter fragment beginning at the *SalI* site (position -226) and extending toward the CAT gene. Plasmid p35K-PRM was in turn used to generate deletion series p($\Delta 3'$ -X)35K-CAT, in which sequences were removed from the 3' side of the promoter fragment beginning from the RNA start site. Plasmid p35K-PRM was originally constructed by inserting the 240-bp promoter fragment from plasmid p35K-CAT/*hr5* that extended from the *SalI* site (position -226) to the *HindIII* site (position +12) into the Bluescript (KS) vector (Stratagene, La Jolla, Calif.). Combinations of 5' and 3' promoter deletions were assembled by inserting the *Bgl*III-*Bam*HI fragment from p($\Delta 5'$ -Y)35K-CAT plasmids that contained the 35K promoter-CAT gene into the unique *Bgl*III site of p($\Delta 3'$ -X)35K-PRM plasmids, thereby generating the plasmid series p($\Delta 3'$ -X/ $\Delta 5'$ -Y)35K-CAT (Fig. 2A).

Site-directed mutagenesis using synthetic oligonucleotides was conducted as described by Kunkel et al. (23). Three oligonucleotides, 5'-GAATATTTATAATATAACCAGATCTGGTACGACGCGGTG-3' (positions -20 to -59), 5'-CCAGAGATAACACACAGATCTGTCACTCATGTTAC-3' (positions -86 to -119), and 5'-CAGAGATAACACACGAGATCTGACTCATGTTACAG-3' (positions -87 to -121), containing a unique *Bgl*III site (underlined) were independently annealed to uracil-containing single-stranded DNA derived by VCSM13 (Stratagene) helper phage rescue of plasmid p35K-PRM from *Escherichia coli* CJ236 (*dut ung*). DNA synthesis was carried out with T7 DNA polymerase (U.S. Biochemical Corp., Cleveland, Ohio) using conditions described previously (41). Oligonucleotide-mediated mutations (and exonuclease III-deletion endpoints) were confirmed by nucleotide sequencing. After mutagenesis, the 35K promoter fragment was fused to the CAT gene for subsequent insertion into the viral genome.

The transplacement vector pEVocc⁺/PA (Fig. 2B) was constructed to accommodate the insertion of foreign DNA, using a polylinker positioned at the *EcoRV* site [3.96 m.u.] immediately upstream from the intact polyhedrin gene. In brief, the promoter and structural portion of the polyhedrin gene, located on a 730-bp *EcoRV*-*KpnI* fragment (derived from the *EcoRI* I genome fragment of AcMNPV), was first

FIG. 2. (A) Strategy for construction of 35K promoter deletion plasmids p(Δ3'-X/Δ5'-Y)35K-CAT. Deletions from the 3' side of the 35K promoter fragment were generated from plasmid p35K-PRM by consecutive processes: restriction with *HindIII* and *PstI*, treatment with exonuclease III (*ExoIII*), and addition of *BglII* linkers (dark box); intramolecular ligation yielded plasmid series p(Δ3'-X)35K-PRM, where X designates the nucleotide position of the 3' endpoint. Deletions from the 5' side of the promoter were generated from plasmid p35K-CAT/hr5 in the same manner except that restriction endonucleases *SstI* and *Sall* were used to produce plasmid series p(Δ5'-Y)35K-CAT, where Y designates the 5' endpoint. Combinations of deletions were then assembled by inserting the CAT-containing *BglII*-*BamHI* fragment from plasmids p(Δ5'-Y)35K-CAT into the unique *BglII* site of plasmids p(Δ3'-X)35K-PRM. The resulting p(Δ3'-X/Δ5'-Y)35K-CAT plasmids contained the indicated 5' or 3' deletions (see text) of the 35K promoter fragment fused to the CAT gene. Thin arrows denote the position of the early RNA start site for the 35K protein gene. Restriction site abbreviations: B, *BamHI*; Bg, *BglII*; H, *HindIII*; K, *KpnI*; N, *NruI*; P, *PstI*; S, *Sall*; Ss, *SstI*; Xb, *XbaI*; Xh, *XhoI*. (B) Structure of the AcMNPV transplacement vector pEV_{occ}⁺/PA. A polylinker with the indicated restriction sites is positioned immediately upstream from the promoter and structural portion (shaded arrow) of the polyhedrin gene; this region is flanked on either side by sequences from 3.2 to 7.1 m.u. of the AcMNPV genome (open boxes). An AcMNPV-derived polyadenylation signal (poly A site) was introduced to promote processing of RNAs transcribed in the counterclockwise direction from the polylinker. Unique restriction sites are indicated by asterisks.

inserted into the *DraII* (previously end repaired with the Klenow enzyme) and *KpnI* sites, respectively, of the Bluescript (KS) vector. A resulting *SstI*-*KpnI* fragment (end repaired at the *SstI* site with T4 DNA polymerase) that contained the polyhedrin gene and promoter was then subcloned into the unique *EcoRV* and *KpnI* sites of plasmid pEV55 (25) in which the *SmaI* and *PstI* sites were previously removed. Lastly, a bidirectional polyadenylation signal, located on a 148-bp *Sau3A*-*HindIII* fragment (derived from 85.1 m.u. of the AcMNPV genome [8, 30]), was inserted next to the polylinker. Transplacement plasmids (pEV_{occ}⁺/PA/35K-CAT) carrying the 35K promoter fused to the CAT gene were constructed by inserting the *XhoI*-*XbaI* fragment from various p(Δ3'-X/Δ5'-Y)35K-CAT plasmids into the corresponding *XhoI* and *XbaI* sites of pEV_{occ}⁺/PA.

Recombinant viruses. AcMNPV recombinants were constructed by using standard gene replacement methods (reviewed in references 24 and 25). pEV_{occ}⁺/PA/35K-CAT plasmid DNA (15 μg), previously linearized by *NdeI* digestion, was mixed with 1 μg of viral DNA and 30 μg of Lipofectin (Bethesda Research Laboratories, Gaithersburg, Md.) and then added dropwise to Sf21 cells (2 × 10⁶ cells per 60-mm-diameter plate) in serum-deficient medium. After a 6-h incubation at 27°C, the medium was removed and replaced with serum-containing TC100 medium. Extracellular virus (approximately 10⁷ PFU/ml) was harvested 3 to 4 days later. The viral DNA used for gene replacement was isolated from an L-1 AcMNPV-deletion mutant (kindly

provided by L. K. Miller, University of Georgia) that lacked the polyhedrin gene and its promoter. Recombinant viruses were identified by screening isolated plaques for the production of viral occlusion particles with sizes and numbers typical of wild-type AcMNPV. After two additional rounds of plaque purification, recombinant viruses were analyzed for the proper insertion of 35K-CAT sequences, including the *BglII* sites specifying the location of promoter mutations, by restriction mapping and Southern blot analysis (data not shown).

CAT assays. Cells were harvested by scraping the monolayer at the indicated times after infection and were then washed and lysed by three freeze-thaw cycles (29). The resulting lysates were assayed for CAT, using [¹⁴C]chloramphenicol (57 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) as described previously (11). To measure the effects of various promoter mutations, duplicate assays were conducted with at least two independent isolates of the same 35K-CAT recombinant virus. The averaged values of CAT activity are reported as nanomoles of chloramphenicol acetylated per minute per 10⁶ cells or as a percentage of that expressed by the indicated reference virus.

Primer extension analysis of RNA. Total RNA was isolated from cells at the indicated times after infection by using the guanidine isothiocyanate-cesium chloride method (4). RNA (50 μg) was annealed to a 39-base oligonucleotide primer that was complementary to the CAT gene (positions +15 to +53 relative to the ATG initiator codon) and previously 5' end

labeled with [γ - ^{32} P]ATP (>5,000 Ci/mmol; Amersham). Primer extension was conducted with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), using conditions described previously (29). The extended products were subjected to electrophoresis on 6% polyacrylamide–8 M urea–TBE (100 mM Tris-borate [pH 8.3], 2 mM EDTA) gels and autoradiography. Dideoxy-chain termination (40) ladders were generated by using [^{35} S]dATP and the above-described CAT-specific primer annealed to double-stranded DNA template prepared from pEVocc⁺/PA/35K-CAT plasmids.

RESULTS

Insertion of 35K promoter mutations into the AcMNPV genome. To identify and map the *cis*-acting regulatory elements that are involved in early transcription of the 35K protein gene, we constructed specific mutations within the upstream promoter region (35K promoter) of this gene and analyzed the ability of the affected promoter to direct expression of the CAT reporter gene. Mutagenesis of transcriptional regulatory elements was expected to alter (either increase or decrease) promoter activity, thereby providing a means by which to ascertain the role of various DNA elements in mediating early viral transcription.

To facilitate incorporation of the 35K promoter fused to the CAT gene (35K-CAT reporter) into the AcMNPV genome, we constructed a modified transplacement vector, pEVocc⁺/PA (Fig. 2B), that employed the occlusion-specific polyhedrin gene as a visual marker for the identification of recombinant viruses. pEVocc⁺/PA contains a polylinker for the insertion of foreign DNA within a nonessential region of the viral genome (6, 10) located immediately upstream from the polyhedrin gene. After cloning of various 35K-CAT reporters in this site, the resulting transplacement plasmids were cotransfected into cultured cells with viral DNA from an AcMNPV-deletion mutant that lacked the polyhedrin gene. Genomic insertion of the 35K-CAT reporter, linked to the polyhedrin gene, was accomplished through allelic replacement in which recombination occurred between regions of vector DNA that flank the polyhedrin gene (Fig. 2B) and the corresponding regions of the viral genome. Recombinant virus plaques that produced wild-type levels of occluded virus particles were visually identified amid the background of less conspicuous, occlusion-negative plaques.

When embedded within the viral genome (Fig. 3A), the full-length 35K promoter, consisting of sequences from positions –226 to +12 (RNA start site, position +1), behaved in a manner indistinguishable from that of the wild-type 35K promoter. Expression of the CAT reporter was detected within 2 h after infection (Fig. 3B); moreover, the maximum rate of CAT accumulation was from 2 to 4 h after infection, paralleling the synthesis of the α_1 mRNA for the 35K protein gene (29). Early 35K-CAT RNAs were initiated from the same site utilized by the wild-type promoter (see Fig. 5). Later in infection, CAT continued to accumulate but at a reduced rate; this late expression was most likely due to low-level transcription from the late 35K promoter element. To ensure that subsequent mutational analyses examined the role of promoter elements in mediating early transcription, assays for CAT expression were conducted on cells 4 h after infection during the peak of early 35K promoter activity.

Identification of early regulatory elements by using progressive 5' and 3' deletions of the 35K promoter fragment. Our previous studies indicated that the 5' boundary of elements mediating early transcription from the 35K promoter was

FIG. 3. (A) Structures of recombinant viruses at the site of genomic insertion of the CAT reporter fused to the 35K promoter. The 35K-CAT reporter, contained on an *XhoI*-*XbaI* fragment from plasmids p(Δ3'-X/Δ5'-Y)35K-CAT (Fig. 2A), was introduced into the AcMNPV genome upstream from the polyhedrin gene (3.96 m.u.). Early transcription of the CAT gene directed by the 35K promoter was from right to left (counterclockwise), opposite that of the adjacent polyhedrin gene. The *BglII* linker (dark box) depicts the position of deletions or altered sequences within the 35K promoter fragment that differs between recombinant viruses. The flanking *SalI* and *HindIII* sites (3.2 and 5.2 m.u., respectively) represent the same sites within the pEVocc⁺/PA transplacement vector (Fig. 2B). Restriction site abbreviations are given in the legend to Fig. 2. (B) Temporal expression of CAT directed by the full-length 35K promoter. Cell lysates were prepared from duplicate cultures at the indicated times after infection with recombinant virus v(Δ3'-162/Δ5'-154)35K-CAT containing the full-length promoter fragment. The level of CAT in each lysate was determined, averaged, and reported as nanomoles of chloramphenicol acetylated per minute per 10⁶ cells.

located between positions –155 and –55 (29). To more precisely define this boundary and to locate individual regulatory elements downstream, we analyzed the effects of four series of deletions (A through D) on the ability of the 35K promoter to direct expression of the CAT reporter early after infection. In deletion series A (Fig. 4A), DNA was removed from the promoter fragment in ~20-bp intervals starting from the 5' side at position –162; all deletions were joined by an 8-bp *BglII* oligonucleotide linker. There was no effect on early CAT expression until sequences residing between –91 and –75 were removed (Fig. 4B, series A); deletion to –75 reduced expression to 34% of that from the full-length promoter of recombinant virus v(Δ3'-162/Δ5'-154)35K-CAT (Fig. 4A, series A), defined throughout this study as having 100% promoter activity. A second reduction to 10% of full activity was observed when sequences between –57 and –30 were deleted and suggested that at least two elements with 5' boundaries near positions –57 and –91, respectively, affected early CAT expression. Further removal of sequences to –23, including the putative TATA box (positions –30 to –25), reduced activity to 4.5%, while deletion to –2, including part of the late 35K promoter element, reduced activity to 2%. This 98% loss of CAT

FIG. 4. Effects of progressive deletions of the 35K promoter on early CAT expression from recombinant viruses. (A) Boundaries of 35K promoter deletions. Four series of deletions (A through D) are depicted below the nucleotide sequence of the wild-type 35K promoter region from position -162 to the early RNA start site (position +1). The endpoints of each deletion are joined by a *Bgl*II oligonucleotide linker (CA GATCTG; striped box) positioned on either side of an open gap of missing nucleotides. Series A ($\Delta 3'-162/\Delta 5'-Y$) deletions extend in the 5' direction from -162 to the nucleotide (Y) indicated on the left. Series B ($\Delta 3'-X/\Delta 5'-30$) and C ($\Delta 3'-X/\Delta 5'-57$) deletions extend in the 3' direction from -30 and -57, respectively, to the indicated nucleotide (X). Series D ($\Delta 3'-X/\Delta 5'-Y$) deletions extend to the nucleotides (X and Y) indicated. Transcriptional control elements defined by this study (see text) are depicted below the sequence of the promoter fragment and include the distal (dis) GC, proximal (prox) GC, distal CGT, proximal CGT, and TATA motifs. Late RNAs were initiated from positions -4 and -5 within the 35K late promoter element (double underline), while the divergent α_4 RNA for the early 94K protein gene initiated near position -105 (8). (B) Relative levels of CAT expressed by recombinant 35K-CAT viruses. Cell lysates prepared early (4 h) after infection with the recombinant viruses of each deletion series (A through D) were assayed for CAT. The level of CAT expressed by each virus, designated by the nucleotide position ($\Delta 5'$, $\Delta 3'$, or both) of the deletion endpoint, is displayed as a percentage of that expressed in the same assay by recombinant virus v($\Delta 3'-162/\Delta 5'-154$)35K-CAT that contains the full-length 35K promoter (designated FL). The reported values with standard deviations (bars) represent the average of duplicate assays conducted on two independently isolated recombinant viruses.

expression upon removal of the sequences comprising the 35K promoter (positions -154 to -2) demonstrated that the promoter fragment itself was responsible for directing most, if not all, of the observed early reporter expression in these analyses.

To further define the distal and proximal regulatory elements, the effects of deletions extending from the opposite (3') direction were examined. In deletion series B (Fig. 4A), DNA was removed from the promoter fragment in the 3' direction beginning immediately upstream (position -30)

from the TATA box. Despite an initial stimulation of CAT expression upon insertion of the *Bgl*II linker (Fig. 4B, series B), deletions extending to -49 and -59 reduced expression to 80 and 45%, respectively, relative to that of the full-length promoter. This result confirmed that one or more elements affecting early promoter activity were located immediately upstream from the TATA box. Further deletion to -91 had little effect, while deletion to -115 reduced expression to 7%, which was similar to that from the promoter fragment containing only the TATA box and sequences downstream (hereafter defined as the basal promoter). This reduction was unexpected, since removal of the same region (positions -91 to -115) from the 5' direction had no effect (Fig. 4B, series A). A possible explanation was that stimulation by this region depends on its spatial relationship to downstream elements (see below); note that deletion from -30 to -91 shifted this region 53 bp downstream to a position immediately adjacent to the TATA box.

To further examine the role of the distal elements (located between -115 and -75), series C deletions (Fig. 4A) were constructed in which promoter sequences were removed in the 3' direction starting at -57 . Removal of DNA between -59 and -75 had no effect on early CAT expression (Fig. 4B), while deletions extending to -115 and -162 reduced activity to 24 and 42%, respectively, compared with the activity of the full-length promoter. Thus, in the absence of the distal elements between -115 and -75 , CAT expression was reduced to a third of that from the full-length promoter, thereby confirming the stimulatory influence of this region. The small but reproducible increase (from 24 to 42%) when sequences between -162 and -115 were deleted suggested that this region contained a potential inhibitory element; it is noteworthy that the α_4 RNA start site and putative regulatory elements for the divergent 94K protein gene lie within this region (Fig. 4A, top).

Transcriptional mapping and quantitation of 35K-CAT RNAs. High-resolution primer extension mapping of early 35K-CAT RNAs (Fig. 5) indicated that in all cases in which the deletions described above did not alter the putative TATA box, transcription initiated from the proper wild-type position (+1). Moreover, all deletions that reduced the level of early CAT expression in infected cells exhibited a parallel reduction in the steady-state level of early 35K-CAT RNA (compare lanes 7 to 12). Upon deletion of the TATA box, transcription from the +1 position was not detected (lane 12); instead, transcription was initiated from other nearby sites, not normally used and revealed only by longer exposures (data not shown). These alternative RNA starts could account for the low level (4.5%) of early CAT expressed by recombinant virus $v(\Delta 3'-162/\Delta 5'-23)$ 35K-CAT that lacked the TATA sequence (Fig. 4, series A deletions).

Analogous to the normal 35K promoter, the 35K-CAT RNA start site shifted late in infection from +1 to a site located just upstream (Fig. 5; compare lanes 5 and 6); this site, TTAAGT (RNA starts underlined; positions -4 and -5 , respectively), resembles the consensus motif (TAAGTA TT) of late and very late baculovirus promoters (33, 37, 38). In contrast to early transcripts, 5' deletions to within 17 bp of the late RNA start site had no effect on the initiation or relative abundance of late transcripts (lane 13). Primer extension of late RNA yielded additional products (unlabeled arrows, lanes 6 and 13) that mapped further upstream at or near the transcriptional initiation site of the divergent polyhedrin gene (Fig. 3A). These products were possibly derived by extension of an RNA encoding the 603-nucleotide open reading frame mapped previously to this location (10)

FIG. 5. Primer extension analysis of 35K-CAT RNAs from recombinant viruses. Total RNA isolated from cells early after infection (4 h) with viruses containing the 35K promoter deletions indicated above the lanes was annealed to a radiolabeled oligonucleotide primer complementary to the CAT gene (positions +15 to +53 relative to the ATG initiator codon). After extension with reverse transcriptase, the products generated from the equivalent of 14 μ g of RNA were subjected to gel electrophoresis and autoradiography. Equivalent amounts of total RNA isolated late after infection (24 h) with recombinant viruses $v(\Delta 3'-162/\Delta 5'-154)$ 35K-CAT and $v(\Delta 3'-162/\Delta 5'-23)$ 35K-CAT (lanes 6 and 13, respectively) were also analyzed. A dideoxy-chain sequencing ladder (lanes 1 to 4) was generated by using plasmid $p(\Delta 3'-162/\Delta 5'-154)$ 35K-CAT and the same primer. The nucleotide sequence comprising the early (E, position +1) and late (L, positions -4 and -5) RNA initiation sites of the 35K promoter are indicated on the right.

or an RNA initiated even further upstream encoding the 1,629-nucleotide open reading frame (32).

Early activating elements are composed of upstream GC and CGT motifs. The deletion analysis described above indicated that early 35K promoter activity was affected by at least two regions located upstream from the TATA box: positions -91 to -75 and -57 to -30 . Examination of the nucleotide sequence within each activating region revealed two types of DNA motifs, designated GC and CGT motifs, respectively (Fig. 4A, top), which because of their repetition and their presence within the upstream promoter region of several AcMNPV genes (29, 43, 44) suggested that they represented potential regulatory elements. To assess the role of each, mutagenesis that was designed to minimize alterations in the spatial arrangement of regulatory sequences within the promoter was used.

FIG. 6. Site-directed mutagenesis of CGT motifs within the 35K promoter. Nucleotide substitutions (highlighted in black), containing a unique *Bgl*/II restriction site, were introduced into the 35K-CAT promoter by oligonucleotide-mediated mutagenesis. The location of the distal and proximal CGT motifs (dis CGT and prox CGT), the distal and proximal GC motifs (dis GC and prox GC), and the TATA box are shown with respect to the wild-type 35K promoter (top). The level of CAT expressed 4 h after infection by the indicated 35K-CAT viruses (left) was determined as described for Fig. 4 and is reported as a percentage of that expressed by the full-length 35K promoter (defined as having 100% activity) of recombinant virus v(Δ 3'-162/ Δ 5'-154)35K-CAT (wt). Also included in this analysis was recombinant virus v(Δ 3'-59/ Δ 5'-48)35K-CAT (Δ pGC), in which the proximal GC motif was replaced by a *Bgl*/II linker.

Two different GC motifs, containing the core sequence GC GCG and located at distal (−83 to −79) and proximal (−56 to −52) positions within these activating regions, were tested for their role in mediating early promoter activity (Fig. 4, series D deletions). Linker replacement of the proximal motif (resulting in only a 2-bp deletion) reduced CAT expression to 21% of full activity [Fig. 4, virus v(Δ 3'-59/ Δ 5'-48)35K-CAT]. A similar reduction to 34% resulted when the distal GC motif was removed [virus v(Δ 3'-91/ Δ 5'-75)35K-CAT]. Each element therefore provided a three- to fivefold increase in early 35K promoter activity; this was confirmed by a larger deletion [virus v(Δ 3'-91/ Δ 5'-48)35K-CAT] that simultaneously removed both GC motifs and resulted in a fourfold reduction in CAT expression (to 28%).

CGT motifs containing the core sequence A(A/T)CGT(G/T) were also identified at proximal (positions −47 to −36) and distal (positions −107 to −102) locations within the 35K promoter (Fig. 6). Oligonucleotide replacements of the proximal element, consisting of tandem CGT motifs (AACGTTG AACGTT), reduced CAT expression to 22% of that from the wild-type promoter (Fig. 6, 35K-CAT virus Prx). In contrast, mutagenesis of the distal CGT motif (ATCGTG) had no effect, as suggested by the near-normal levels of expression by two different recombinant viruses (Dis1 and Dis2). Simultaneous replacement of the distal and proximal CGT motifs (35K-CAT virus Dis1/Prx) reduced expression to a level (17%) similar to that when the proximal region alone was mutated. These results indicated that only the proximal motifs affected promoter activity in the context of the full-length promoter. While the distal CGT motif had no apparent effect in its normal position, internal deletions that placed this motif adjacent to the TATA box demonstrated that it could provide a comparable fivefold stimulation of early promoter activity in the absence of the proximal CGT motif [Fig. 4; compare viruses v(Δ 3'-91/ Δ 5'-30)35K-CAT and v(Δ 3'-115/ Δ 5'-30)35K-CAT of series B deletions].

Independent mutagenesis of the proximal CGT and GC motifs indicated that each provided a similar contribution to early promoter activity; linker substitutions reduced expression to 22% in both cases (Fig. 6; compare viruses Prx and Δ pGC). While the proximity of the GC and CGT motifs to each other suggested the possibility of overlap, the stepwise

reduction in CAT expression (from 140% to 80 and 45%, respectively) that resulted when the sequences encompassing each motif were sequentially removed argued that each represents a distinct regulatory element (Fig. 4, series B deletions). More precise mutagenesis will be required to define the exact boundary of each motif at this location.

It is noteworthy that during the course of isolating the recombinant viruses (Fig. 6), we encountered a significant instability of the linker-induced mutations, particularly those that were centered within the 35K promoter fragment. Southern blot analysis (not shown) revealed that in many cases, a plaque-purified recombinant virus contained a mixture of genotypes in which a portion (1 to 20%) of the virus population lacked the newly introduced *Bgl*/II linker. Accompanying this loss was the appearance of a *Bgl*/II site within the homologous wild-type promoter of the 35K protein gene, also present in the genome but located over 20 kb away. This finding suggested that the disappearance of the mutation was due to recombination that exchanged sequences between the 35K-CAT promoter and the normal 35K promoter. Since instability of a mutation within a regulatory motif would affect the overall level of 35K-CAT expression, recombinant viruses were carefully screened such that only those viruses that exhibited less than a 5% loss, if any, of the linker mutation were analyzed; thus, any effect on the level of CAT expression was less than the standard deviation for the assay itself (averaging $\pm 10\%$). These preliminary observations suggested, nonetheless, that recombination may lead to substantial instability of repetitive DNA introduced into the AcMNPV genome and warrants attention during construction of recombinant viruses.

The TATA box represents the basal promoter element for early transcription of the 35K protein gene. Mutagenesis of the upstream promoter region (positions −90 to −30) reduced but never abolished 35K promoter activity. Not until the putative TATA box (−30 to −25) was deleted was transcription from the proper RNA start site eliminated (Fig. 5). This suggested that the TATA element (possibly in combination with downstream elements) constituted the basal (minimum) promoter for the 35K protein gene. To examine the role(s) of the TATA element, the effect of

FIG. 7. Mutagenesis of the TATA box and the early RNA start site of the 35K promoter. (A) Locations of site-directed mutations and effects on CAT expression. Positions of the TATA box (rectangle; position -30) and RNA start site (horizontal arrow; position +1) are indicated within the sequence of the wild-type (wt) 35K promoter (top). The TATA element of 35K-CAT viruses v(Δ3'-32/Δ5'-23) and v(Δ3'-36/Δ5'-23) was replaced by a *Bgl*III linker (underlined). In contrast, 35K-CAT viruses v(Δ3'-7/Δ5'-23), v(Δ3'-7/Δ5'-20), v(Δ3'-11/Δ5'-20), and v(Δ3'-11/Δ5'-23) contain insertions (highlighted in black) of 25, 22, 18, and 21 nucleotides, respectively, positioned between the TATA box and the RNA start; these insertions were generated by joining the indicated 5' and 3' promoter deletions with a *Bgl*III linker. Solid vertical arrows mark the positions of RNA start sites mapped in panel B; dashed arrows mark the RNA start sites mapped by similar primer extension analyses (data not shown). The level of CAT expressed 4 h after infection with the indicated 35K-CAT viruses is reported as a percentage of that directed by the full-length promoter of recombinant virus v(Δ3'-162/Δ5'-154)35K-CAT (FL). (B) Mapping of the 5' ends of early 35K-CAT RNAs from insertion mutants. Primer extension analysis of total RNA isolated 4 h after infection with recombinant 35K-CAT viruses containing the full-length wild-type [FL (wt)] promoter (lane 5) or the indicated insertion mutants (lanes 6 to 9) was conducted as described for Fig. 5, using the same 39-base CAT-specific primer. Dideoxy sequencing ladders were generated from plasmids p(Δ3'-162/Δ5'-154)35K-CAT (lanes 1 to 4) and p(Δ3'-7/Δ5'-23)35K-CAT (lanes 10 to 13), using the same primer.

site-directed mutations from positions -30 to +1 was assessed by using recombinant 35K-CAT viruses.

Exact replacement of the TATA sequence with an oligonucleotide linker resulted in a 20-fold reduction in early CAT expression (Fig. 7A); expression was 7% [recombinant virus v(Δ3'-32/Δ5'-23)35K-CAT] of that from the full-length, wild-type promoter (100%). This was confirmed by the removal of four additional nucleotides from the upstream side of the TATA element, which reduced activity to 5% [virus v(Δ3'-36/Δ5'-23)35K-CAT]. That both mutations produced the single largest reduction in expression indicated that the TATA element is a critical component of the early promoter.

Primer extension mapping (data not shown) indicated that disruption of the TATA element resulted in the initiation of 35K-CAT transcripts from alternative sites (Fig. 7A, dashed arrows). To therefore determine whether the TATA element functioned to direct transcription from a site located a specified distance downstream, recombinant viruses were constructed in which DNA (18 to 25 bp) was inserted between the TATA box and the normal start site, thereby separating both elements without altering the sequence of either. Primer extension analyses (Fig. 7B) demonstrated that in each of four insertion mutants examined, the distance between the TATA element and the utilized RNA start site was conserved; new transcripts (solid vertical arrows in Fig. 7A) always initiated ~30 bp downstream from the TATA sequence, and in no case was transcription detected from the normal RNA start site located further downstream. Thus, the sequences comprising the TATA box contain the information necessary for establishing the location of the early RNA start site.

Closer examination of the RNA start sites of these insertion mutants suggested that certain nucleotides may be

preferred for initiation although they must lie within a window for initiation dictated by the TATA element. For example, virus v(Δ3'-11/Δ5'-20)35K-CAT initiated RNA from a single A residue (Fig. 7A, asterisk) located exactly 30 bp downstream from the TATA box. RNAs were initiated from this same residue despite its location 3 bp further downstream within virus v(Δ3'-11/Δ5'-23)35K-CAT. However, as this residue was moved even further downstream relative to the TATA box, initiation decreased or disappeared, as shown by viruses v(Δ3'-7/Δ5'-20)35K-CAT and v(Δ3'-7/Δ5'-23)35K-CAT, respectively (Fig. 7B); heterogeneous start sites were used by both of these viruses. The sequence at the RNA start site also influenced the level of CAT expression. Recombinant virus v(Δ3'-7/Δ5'-23)35K-CAT expressed nearly normal levels of CAT (118%) despite a very different sequence at its RNA start site compared with the wild-type promoter (Fig. 7A). In contrast, recombinant virus v(Δ3'-7/Δ5'-20)35K-CAT, which was identical to the previously mentioned virus except for three nucleotides (ATT) missing from the RNA start site, exhibited a three- to fourfold reduction in expression. Similar reductions were observed with the other insertion mutants (Fig. 7A). Whether these changes in expression levels were due to sequence differences at the RNA start site that affected transcription or the stability of 35K-CAT mRNAs with differing 5' ends remains to be determined.

DISCUSSION

General organization of the 35K gene promoter as analyzed in the context of the viral genome. When introduced at an alternate location within the AcMNPV genome (3.96 m.u.), the 35K promoter fragment (positions -226 to +12) directed

transcription from the same sites and with the same temporal specificity as when located at its normal position (Fig. 5). This made it possible to use site-directed mutagenesis to identify *cis*-acting promoter elements mediating temporal regulation during a normal infection. This approach was expected to provide the most precise analysis of early transcriptional regulatory elements; indeed, we previously noted substantial differences in the role of upstream promoter elements when assayed by transient expression in the presence of cloned stimulatory elements such as IE1 (29). The strategy used in this study for insertion of the 35K-CAT reporter into the viral genome was designed to avoid the deletion of viral DNA, including the polyhedrin gene, which provided a convenient means for identifying viral recombinants. This minimized potential mutagenic effects on local viral genes, which could have altered expression of the CAT reporter; mutations that affect polyhedrin transcription, for example, alter the levels of overlapping, antisense RNAs within the region (31). It may be relevant in this regard that 35K-CAT expression was two- to threefold higher in cells infected with recombinant viruses expressing normal levels of polyhedrin than in those used previously that lacked the polyhedrin gene (29).

Our analysis of a variety of mutations indicates that the promoter that directs early transcription of the 35K protein gene consists of a basal (minimum) promoter element and an upstream activating region (UAR). The TATA element constitutes the basal promoter and is essential for early promoter activity. The UAR, located immediately upstream (positions -90 to -30) from the TATA element, is composed of individual regulatory motifs that potentiate the transcriptional activity of the basal promoter. The organization of the early 35K promoter is therefore typical of promoters (both host and viral) that are responsive to RNA polymerase II (for recent reviews, see references 26 and 28); indeed, transcription of the 35K protein gene is mediated by the host cell RNA polymerase II as suggested by its sensitivity to α -amanitin (18). In contrast to early transcription, late transcription was unaffected by mutagenesis of the UAR or the TATA element (Fig. 5). Deletion of the early regulatory elements, in effect, converted temporal expression of the 35K promoter to that of a late viral gene. Late expression was eliminated only when deletions removed the late promoter motif TTAAGT (RNA starts underlined) that resembles the major sequence determinant for late and very late baculovirus promoters (37, 38). This finding supports the contention that the *cis*-acting elements mediating early and late viral transcription function independently despite possible overlapping sequences.

Role of the TATA element in early basal transcription. Mutagenesis of the TATA box (positions -30 to -25) resulted in a 20-fold reduction in early 35K promoter activity (Fig. 7); no other single element exhibited as strong of an influence on early activity. In addition, mutagenesis upstream or downstream of the TATA element altered but never abolished early promoter activity. These results support the contention that the TATA box constitutes the basal promoter which is influenced by auxiliary regulatory elements. Two lines of evidence suggested that another role of the TATA element is to establish the position of the RNA start site. First, linker replacement of the TATA element resulted in the appearance of alternative transcriptional start sites. Second, upon insertion of DNA between the TATA box and the normal RNA start site, the distance to the utilized RNA start site remained constant (29 to 34 bp, representing about three turns of the DNA helix) (Fig. 7).

The observation that the sequence at the new initiation sites differed and that transcription was never detected from the normal α_1 RNA start site (Fig. 7) argued against the involvement of an initiator motif (27, 36, 42) that establishes the transcription start. Rather, our data indicated that the sequences comprising the TATA element contain all information necessary to direct proper downstream initiation. The initiation sequence of the 35K promoter differs significantly from that of several early baculovirus genes (2) that contain a conserved CAGT motif which matches a larger consensus ATCA(G,T)I(C,T) at the same position within many insect promoters (19). While this sequence functions in the absence of a TATA box as a transcription initiator in *Drosophila* cells (21), such a role in early baculovirus transcription has yet to be demonstrated.

The requirement of the TATA element for 35K promoter activity and the observation that identical (TATAA) or closely related motifs are found approximately 30 bp upstream from the RNA start site of many early AcMNPV genes (2, 5, 8, 12, 14, 22, 34, 35, 43, 44) suggested that this element is a crucial component of most, if not all, early baculovirus promoters. This predicts that a TFIID-like host factor is involved in regulating early promoter activity. As part of the general transcription complex, TFIID mediates basal transcription by binding to sequences that include the TATA box and is the target of activation by auxiliary transcription factors (16). Several virus-encoded *trans* activators, including adenovirus E1A, herpes simplex virus ICP4, and pseudorabies virus IE protein, stimulate transcription, possibly by direct interaction with TFIID or indirectly through other activators that influence TFIID (1, 20, 46). The mode of interaction of analogous AcMNPV *trans* activators, including IE1, with early promoter elements remains to be determined.

Organization of the 35K promoter UAR: regulatory GC and CGT motifs. Deletion mutagenesis demonstrated that the 60-bp region (-90 to -30) immediately upstream from the TATA element, defined as a UAR, was responsible for a 10- to 15-fold enhancement of transcription from the early +1 start site of the 35K basal promoter (Fig. 4 and 5). Supporting the separability of the UAR from the basal promoter was the preliminary finding (39) that the UAR of the 35K promoter could be functionally replaced with an AcMNPV enhancer (*hr5*) as well as the UAR from another early promoter (AcMNPV DNA polymerase gene); such substitutions restored normal early CAT expression to 35K-CAT viruses that had been deleted of the 35K UAR. Since the *hr5* enhancer increases the rate of transcription from certain viral promoters (13), the 35K UAR may function in an analogous manner although conceivably in response to different *trans*-acting factors. The orientation independence, if any, of UAR stimulation remains to be determined.

Mutagenesis that preserved (or minimally changed) the spatial arrangement of promoter DNA indicated that distinct elements were responsible for the stimulatory activity of the 35K UAR. The most distal element, designated a GC motif (GCGCGATA), was identified between positions -90 and -75; removal of this distal motif reduced activity to 34% of the full-length promoter (Fig. 4). Another GC motif (GCGC GTCG) was identified further downstream between -56 and -49; mutagenesis of this proximal motif reduced activity ~5-fold (Fig. 6) and indicated that both motifs were necessary for full promoter activity. Interestingly, the two GC motifs are nearly identical to the duplicate E2F binding sites, GCGCGAAA (distal GC motif matches underlined), located within the adenovirus E2 promoter (28).

A second type of regulatory element that contained the core consensus A(A/T)CGT(G/T), designated a CGT motif, was also identified on the basis of its effect on early promoter activity. Oligonucleotide-mediated mutagenesis of a CGT motif that consisted of tandem AACGT repeats located immediately upstream from the TATA box (−47 to −36) reduced activity to about 20% of the wild-type promoter (Fig. 6); thus, this region by itself provided a fivefold stimulation of transcription. Further analysis will be required to determine whether both of the core CGT repeats are required for activation and to what extent this motif overlaps the adjacent GC motif (position −54). A third CGT motif (position −104) had little if any effect on early activity in the context of the full-length promoter (Fig. 6). Nonetheless, internal deletions that juxtapositioned the distal CGT motif with the TATA element (thereby replacing the proximal CGT motif) demonstrated that this motif also has the potential to contribute a similar fivefold stimulation (Fig. 4). Interestingly, no stimulatory effect was observed when similar sequences, representing potential CGT motifs for the divergent 94K protein gene and therefore oriented in the opposite direction, were shifted next to the TATA box of the 35K promoter [Fig. 4, virus v(Δ3′-115/Δ5′-30)35K-CAT]. Thus, CGT motif activation may be both position and orientation dependent.

The presence of CGT- and GC-like motifs within the upstream region of several early AcMNPV genes (29, 43, 44) suggests that these elements may play a similar role in activating other early promoters. It is expected that these motifs represent the DNA recognition sites for host or virus-encoded factors that are potential candidates for interaction with the basal transcription apparatus including TFIID. The UAR of early promoters (along with enhancer elements) may recruit combinations of *trans*-acting factors to stimulate host RNA polymerase II-mediated transcription and thereby define the relative strength of a promoter. Such regulatory motifs may also influence virus host range, since transcription factors from the host cell may contribute to providing an adequate level of expression of those genes that are critical for initiating productive replication at a time early in infection when the concentration of viral DNA template is low. Preliminary experiments have indicated that both the level and timing of expression of the early 35K protein gene are crucial for normal AcMNPV replication in cultured *S. frugiperda* cells (39). Thus, further studies on the regulation of this gene may provide insight into the functional importance of early activating elements during viral replication.

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