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Regulation of Delayed-Early Gene Transcription by Dual TATA Boxes

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The 39K *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) gene is highly expressed throughout the virus life cycle and is controlled by tandem promoters that exhibit features of early and late baculovirus promoters. Late transcripts initiate at a conserved TAAG motif, while early transcripts are heterogeneous and initiate near a conserved CAGT motif. To define the nucleotide sequences that regulate early transcription of the 39K gene, a series of mutations was generated by substitution of 10-bp stretches in the 39K promoter with a *Bgl*II linker. The effects of these mutations on transcription from the early promoter were determined by transient expression and primer extension assays in the presence of the viral *trans*-activator IE1 gene. Mutations in the region from −15 to −44 revealed that early 39K transcription was controlled by dual TATA boxes. These TATA boxes are separated by 10 bp, which partially accounts for the heterogeneity in early 39K transcripts. Transcripts initiating at the CAGT motif (proximal transcripts) were abolished by deletion of the proximal TATA box located at −29 relative to CAGT. Proximal transcripts were not affected by alterations in the distal TATA motif located at −39 relative to the CAGT. Similarly, transcripts initiating upstream of CAGT (distal transcripts) were eliminated by mutations in the distal TATA but were unaffected by substitutions in the proximal TATA box. Proximal transcripts were not detected with a plasmid containing mutations in the CAGT motif, although the distal transcripts were unaffected by CAGT mutations. When the sequences surrounding the initiation site for the distal transcripts were altered, the start site was shifted one nucleotide, but transcription was not quantitatively affected. These results suggest that early 39K transcription is controlled by two distinct TATA elements, one that is dependent on an initiator and one in which the site of initiation is determined by the TATA element alone. Mutations in an upstream region from −45 to −68 relative to the CAGT motif had a quantitative effect but did not alter the heterogeneous pattern of early transcripts, suggesting these sequences function as an upstream regulatory region. Analysis of late transcription indicated that the TAAG element was essential, while transcription was unaffected by other mutations.

In cells infected with *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV), genes are expressed in a temporally controlled and sequentially ordered fashion (for a review, see reference 2). These genes have been classified as early, late, and very late, based on their requirements for de novo protein synthesis and viral DNA replication. The early genes may be further divided into immediate early and delayed early, based upon transient assay experiments showing that the viral *trans*-activator IE1 and a viral enhancer element are required for full expression (15, 17). The delayed-early gene products include several viral proteins apparently required for viral DNA replication (9, 27, 39). Late gene expression is concomitant with the onset of DNA synthesis. During the late phase, the structural proteins of extracellular virus are synthesized. The very late gene products are required for the occlusion process and are maximally expressed during this phase.

Most evidence suggests that the cascade of viral gene expression is regulated at the level of transcription. Baculovirus immediate-early and delayed-early genes have promoter elements similar to those from eukaryotic organisms, and transcription is sensitive to alpha-amanitin, suggesting that early genes are transcribed by the host RNA polymerase II (12, 20). Several reports that define regions of early promoters required for transcription have recently been published, although mechanisms of action are largely unknown (3, 10, 38). Transcription of several immediate-early and delayed-early baculovirus genes initiates within a conserved CAGT motif (1, 6, 8, 14, 17, 25). The exact role of this motif in baculovirus transcription is unknown. Mutagenesis of the CAGT motif in the IEN promoter resulted in a threefold decrease in IEN expression, although it was not determined whether the mutations affected transcription, stability, or translation of the message (6). Transcription of late baculovirus genes is resistant to alpha-amanitin (20), suggesting that these genes are transcribed by a virus-encoded or virus-modified host RNA polymerase. In addition, all late genes identified to date contain the consensus late promoter element, TAAG (32). This motif appears to function as both a promoter and an mRNA start site (30, 31). The presence of minicistrons upstream of some baculovirus protein-coding regions suggests that translational regulation may also provide a second level of control (1, 14, 23).

Many of the baculovirus regulatory genes and elements were discovered by using a transient assay system based on the Ac*M*NPV 39K promoter. The 39K gene is highly expressed during the virus life cycle and is controlled by tandem promoters that exhibit features of early and late baculovirus promoters (14, 15). Recently, we have shown that the product of the 39K gene is a 31,000-molecular-weight protein (p31) that is associated with the nuclear matrix, although the function of this protein in the virus life cycle is unknown (14, 41). To map the viral factors that regulate early gene transcription, a recombinant plasmid that contained the reporter chloramphenicol acetyltransferase

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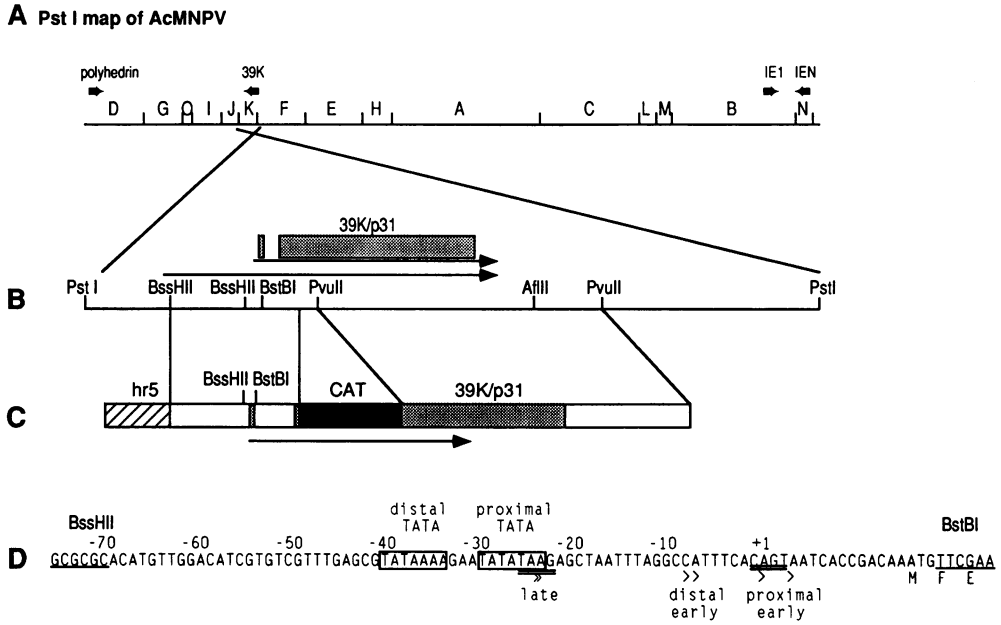

FIG. 1. Location and transcription of the Ac*M*NPV 39K/p31 protein gene. (A) Linear map of the Ac*M*NPV genome showing the *Pst*I restriction fragments. The p31 gene is encoded by the *Pst*I K fragment (21.2 to 23.5 map units) of the wild-type Ac*M*NPV genome. (B) Open reading frame and transcription map of p31 showing the minicistron, overlapping transcripts, and relevant restriction sites. p31 coding regions are shaded. (C) Diagram of the p39LS-WT construct. The CAT gene (black box) was fused immediately adjacent to the methionine codon for p31 (15). The p31 coding regions are shaded, and the enhancer *hr5* is indicated by diagonal stripes. (D) Nucleotide sequence of the region analyzed. The promoter region analyzed in these studies extends from the *Bss*HII site to the *Bst*BI site (positions −73 to +24, relative to the A of the CAGT motif). The p31 gene is transcribed from right to left on the genomic map, so this sequence is reversed relative to genome sense. The 5′ ends of proximal and distal early transcripts are indicated by single arrowheads, and the 5′ end of late transcripts is indicated by a double arrowhead. The two TATA boxes are indicated by rectangles, the CAGT and TAAG motifs are double underlined, and the predicted translation products for the minicistron in the 39K leader are indicated below the sequence.

(CAT) gene under the control of the delayed early 39K promoter was constructed. By cotransfecting uninfected cells with the 39CAT plasmid and various restriction fragments of the Ac*M*NPV genome, the IE1 gene was identified as a viral transactivator (15). Expression of 39K was further increased by cotransfection of the IEN gene, which encodes a protein that stimulates expression of IE1 (5, 6). Cotransfection mapping was also used to define the enhancer function of the homologous DNA regions interspersed in the Ac*M*NPV genome (16). Expression of 39CAT was enhanced 1,000-fold when *hr5* was *cis* linked to the 39K promoter. Recently, we have demonstrated that the IE1 gene product forms a complex in vitro with the *hr5* enhancer (13, 24). Using a more sensitive CAT assay, Theilmann and Stewart (38) could detect low levels of expression from the 39K promoter in uninfected cells, indicating that host factors alone may be sufficient for a basal level of transcription. However, their data confirm our results, which indicate that both IE1 and an enhancer are required for full expression of 39K.

To further understand the mechanisms that regulate 39K transcription, we have conducted a mutational analysis of the 39K promoter. The results presented here reveal that early 39K transcription is controlled by dual TATA elements. One TATA box directs transcription initiation independently of specific sequences at the start site, while the other requires a specific initiation sequence. Transcription from the late 39K promoter is absolutely dependent upon a TAAG motif at the transcription start site.

MATERIALS AND METHODS

Recombinant plasmids. The linker insertion plasmids constructed for this study were derived from plasmid pAc3916, which has been previously described (15). This plasmid contains the bacterial CAT gene cloned in frame with the initiation methionine codon for p31 translation. To construct the parental plasmid p39LS-WT, the baculovirus enhancer element *hr5* was first cloned into the *Pst*I and *Hin*dIII sites upstream of the 39K gene. Then, to eliminate promoter occlusion effects due to transcription from an upstream promoter, the resultant plasmid was digested with *Pst*I and subjected to partial digestion with *Bss*HII (Fig. 1B). The 5′ and 3′ ends were removed with exonuclease VII, and the resulting blunt ends were ligated (33). This plasmid, p39LS-WT, was used to generate 5′ deletions in the promoter region from the unique *Bss*HII site and 3′ deletions from the *Bst*BI site. The deletions were constructed by using the unidirectional exonuclease III procedure (19), followed by the addition of 10-bp *Bgl*II linkers (5′-GAAGATCTTC-3′). Combinations of 5′ and 3′ promoter deletions were generated by inserting the *Bgl*II-*Bst*BI fragments from the 5′ deletions into the *Bgl*II-*Bst*BI sites of the corresponding 3′ deletions. Some mutants were constructed by using synthetic oligonucleotides. The final constructs were sequenced on both strands, using the chain termination procedure (34).

CAT assays and primer extension analysis. The conditions for cell culture, transfections with plasmid DNAs, and CAT assays have been described previously (15, 37). To measure

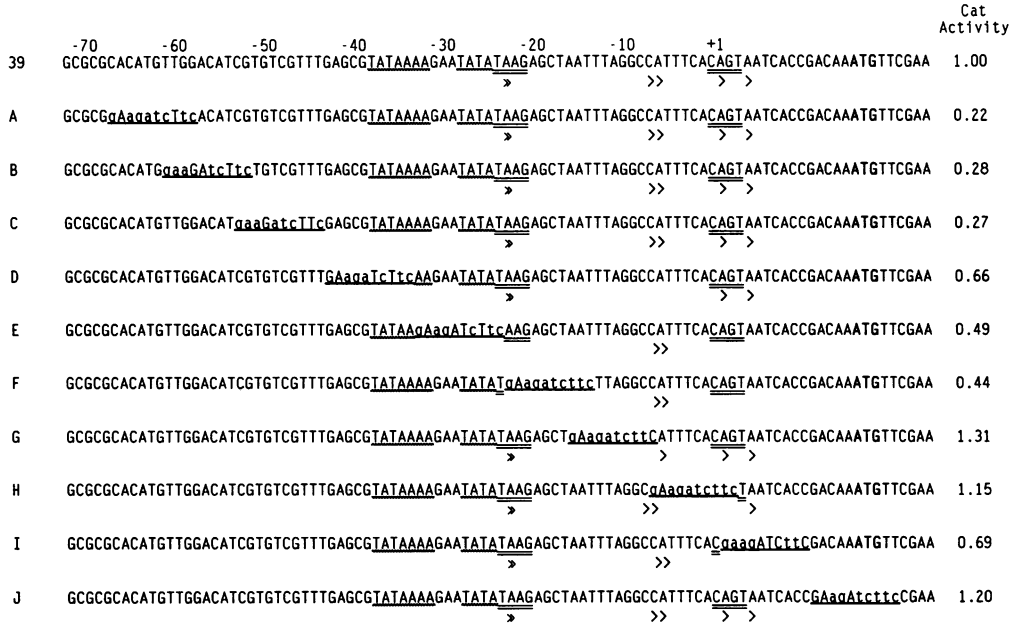

FIG. 2. Summary of LS analyses. The exact nucleotide sequence of each linker insertion mutant is shown. The *Bgl*II sites are underlined; changed nucleotides are shown in lowercase, while the conserved bases are in uppercase. The 5′ ends of early (single arrowheads) and late (double arrowheads) transcripts are shown below the sequence for each mutant. The two TATA boxes are indicated by a dotted underline, the CAGT and TAAG motifs are double underlined, and the ATG codon for the minicistron in the 39K leader is indicated in bold. The CAT activities relative to plasmid p39LS are shown on the right. The level of CAT activity is a function of early gene expression only.

the effects of linker scan (LS) mutants on CAT activity, two different plasmid preparations were assayed in duplicate. The averaged values of CAT activity are reported as a percentage of that expressed by the parental plasmid, p39LS-WT. To measure specific transcripts, total RNA was isolated by the guanidine isothiocyanate-cesium chloride method (7). RNA was annealed to 5′-end-labeled primers, and primer extension was conducted by using avian myeloblastosis virus reverse transcriptase (33). The extended products were analyzed on 6% polyacrylamide–8 M urea gels. Sequencing ladders were generated using p39LS DNA and the same oligonucleotide primers.

RESULTS

Transient assay of p39LS mutants. To identify promoter sequences that serve as transcriptional control signals, LS mutants of the 39K promoter were constructed. The wild-type template, p39LS-WT, that was used to generate deletion mutants contains 380 bp of upstream sequences (Fig. 1C). The construct also contains the entire 39K mRNA leader, including the upstream minicistron, and the CAT gene is fused in frame immediately after the ATG for the p31 open reading frame. Early transcripts initiating from the 39K promoter are heterogeneous in length. To standardize this analysis, +1 is defined as the A nucleotide in the conserved CAGT sequence (Fig. 1D). This sequence is located at the transcription start sites of several baculovirus early genes (2). The construction of LS mutants was limited to a 96-bp region from −73 to +23 relative to the transcriptional start site and includes the ATG of the minicistron. This region also contains the late 39K promoter, which is located at −25 relative to the CAGT motif and overlaps the proximal TATA box. The complete sequences of all LS mutants are presented in Fig. 2.

To analyze the effects of the linker insertion mutations on early transcription, the series of LS mutants was subjected to transient CAT assays (Fig. 3). *Spodoptera frugiperda* cells were cotransfected with IE1 and individual LS mutants. We have previously shown that under these conditions, IE1 transactivates early 39K transcription but does not activate transcription from the late promoter (16). The results of this analysis revealed that mutants p39LS-A through p39LS-F, with substitutions upstream of the tran-

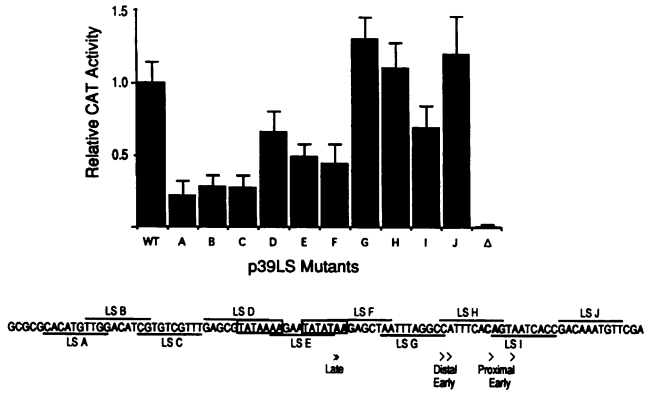

FIG. 3. Effects of linker insertions in the 39K promoter on early CAT expression. *S. frugiperda* cells were transfected with 1 μg of the indicated plasmids and 0.01 μg of IE1. Extracts were prepared 24 h posttransfection and assayed for CAT activity. CAT activity with the mutant plasmids is expressed as a fraction relative to the level obtained with the wild-type plasmid. Values represent the means of eight assays conducted by using two different plasmid preparations; bars indicate standard deviation. The sequence of the promoter region and the locations of the *Bgl*II linkers in the mutant promoters are indicated below.

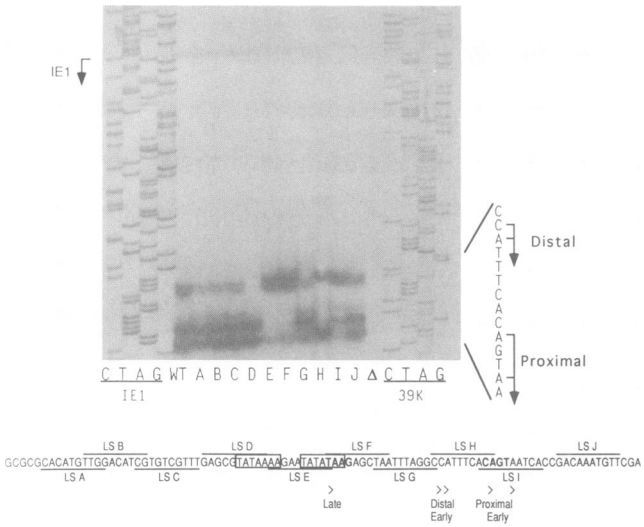

FIG. 4. Primer extension analysis of early 39K RNAs from LS mutants. Cells were transfected with the indicated LS mutants and IE1 as described for Fig. 3. Total RNA was isolated 24 h posttransfection, and 20 μg was annealed to radiolabeled oligonucleotide primers complementary to the CAT gene and the IE1 gene. Sequencing ladders for CAT and IE1 were generated by using the same oligonucleotide primers.

scription initiation sites, had lower levels of CAT activity than did p39LS-WT. Two plasmids, p39LS-H and p39LS-I, contained altered sequences at the transcription initiation sites. p39LS-H exhibited wild-type levels of CAT activity, while p39LS-I showed a 40% reduction in expression of CAT. Plasmid p39LS-J, which alters the methionine codon of the minicistron in the 39K leader, also expressed wild-type levels of CAT. A plasmid with the sequences between *Bss*HII and *Bst*BI deleted, p39LS-Δ, did not induce detectable levels of CAT activity.

Primer extension mapping of early p39LS transcripts. To examine the effects of linker insertions on specific transcripts, primer extension mapping of p39 RNAs was conducted. *S. frugiperda* cells were cotransfected with pIE1 and individual p39LS plasmids. In cells transfected with p39LS-WT, the pattern of transcripts is heterogeneous (Fig. 4). Three sets of transcripts were detected. Two initiation sites mapped near the CAGT motif, and collectively they are referred to as the proximal transcripts. A doublet of transcripts maps to a location at −7 and −8 upstream of the CAGT motif. These are referred to as the distal transcripts. Analysis of p39LS mutants indicated that the relative levels of CAT activity were a function of multiple effects on the pattern of transcripts.

Three of the mutant plasmids, p39LS-A, -B, and -C, had similar patterns of transcription. These plasmids have mutations in an upstream region from −45 to −68. Transcription from these plasmids exhibited the same heterogeneous pattern as seen with the parental plasmid. The level of all transcripts was reduced proportional to the decrease in CAT activity shown in Fig. 3.

Three of the LS plasmids have mutations in either the distal or proximal TATA box. The distal TATA motif is located at −37 relative to the CAGT motif but is located at −32 relative to the distal start sites. p39LS-D contains mutations from −35 to −44, which alters the distal TATA box but does not affect the proximal TATA box. With this plasmid, transcription from the distal start site was eliminated, while the proximal transcripts were unaffected. The transcription pattern is consistent with the CAT activity results, which indicates that the activity was reduced approximately 40%. The opposite result was seen with p39LS-E and p39LS-F. These plasmids have mutations in the region from −15 to −35, so both plasmids have substitutions in the proximal TATA box. Primer extension analysis indicated that transcription from the proximal start site was abolished, while distal transcripts were unaffected. This pattern is also consistent with the CAT activity results presented in Fig. 3, showing that the overall level of expression was reduced approximately threefold.

Plasmid p39LS-G has alterations from −8 to −17, which is between the TATA boxes and the initiation sites. Transient CAT assays with this plasmid indicated that overall expression was slightly higher than that of p39LS-WT. Primer extension analysis revealed a heterogeneous transcription pattern similar to that seen with p39LS-WT.

Two plasmids, p39LS-H and -I, contained substitutions at the proximal and distal start sites. p39LS-H has mutations in the region from +2 to −7. These mutations alter sequences at both initiation sites. However, primer extension data indicate that the effect of these mutations was quite different at the two sites. Transcription from the distal site was shifted upstream one nucleotide, and the level of transcript was comparable to the wild-type level. The proximal transcripts initiating at the CAGT motif were abolished, while those initiating immediately downstream of CAGT were unaffected. Plasmid p39LS-I contains mutations in the region from +1 to +10. Primer extensions revealed that transcription from the proximal sites are significantly decreased with this plasmid, while distal transcription is unaffected. This result is consistent with the twofold reduction in CAT activity (Fig. 3).

Plasmid p39LS-J contains mutations in the region from +11 to +20. This eliminates the ATG codon in the minicistron located upstream of the p31 open reading frame. Transcription was unaffected by this mutation; the same result was seen for expression of CAT (Fig. 2).

Primer extension mapping of late p39LS transcripts. To determine the effects of linker insertion mutations on transcription from the late promoter, p39LS reporter plasmids were cotransfected with wild-type viral DNA and total cell RNA was prepared at 48 h posttransfection (Fig. 4). The RNA was hybridized with oligonucleotides specific for reporter plasmid 39K transcripts. Results of this analysis indicated that transcription from the late p39 promoter was abolished in p39LS-E and p39LS-F. Both of these plasmids contained substitutions in the TAAG motif. Late transcription was not affected in the other plasmids. The level of early transcripts were unaffected by the absence of late transcription, indicating that promoter occlusion is probably not responsible for the regulation of early transcription (11).

DISCUSSION

Our analysis of the insertion mutations in the 39K promoter region indicates that early transcription is controlled by two TATA elements and an upstream regulatory region. Alterations in the upstream regulatory region resulted in a decrease in the level of transcription but did not affect the initiation sites. This region contains one copy of the CGT motif (A[A/T]CGT[G/T]) and one copy of a GC motif, recently described by Dickson and Friesen (10). The GC motif was mutated in LS-A, and the CGT sequence was

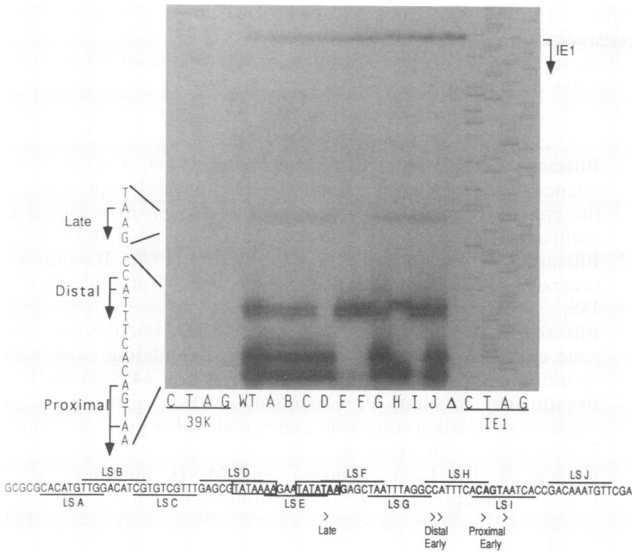

FIG. 5. Primer extension analysis of late 39K RNAs from LS mutants. Cells were transfected with 1 μg of indicated LS mutants and 1 μg of viral DNA as described for Fig. 3. Total RNA was isolated 48 h posttransfection, and 10 μg was annealed to radiolabeled oligonucleotide primers complementary to the CAT gene and the IE1 gene. Sequencing ladders for CAT and IE1 were generated by using the same oligonucleotide primers.

altered in both LS-B and LS-C; these mutants exhibited four- to fivefold decreases in activity, respectively.

Mutational analysis of the two TATA elements indicates that they function independently (Fig. 2). Mutation LS-D, which alters the distal TATA element, abolished transcription initiating from the distal start sites, without affecting transcription from the proximal sites. Mutant plasmids LS-E and LS-F contained substitutions in the proximal TATA box. In cells transfected with these plasmids, transcripts originating from the proximal site were not detected. However, these mutations did not affect transcription from the distal site. The presence of two functional TATA elements is unusual in that most eukaryotic genes contain a single functional TATA element. Another exception is the yeast gene *CYC1* (26). The *CYC1* promoter also contains two functionally independent TATA elements which may be recognized by different transcription factors.

Analysis of mutations at the transcription start sites suggests that the two TATA elements direct transcription by different mechanisms. Mutations at the distal initiation site (p39LS-H) did not affect the level of steady-state transcripts, although the start point was shifted by one nucleotide. This is an expected result for a eukaryotic gene that contains a TATA box (4). TATA boxes are usually the primary determinant of the RNA start site, and transcription initiation is relatively unaffected by sequence changes at the start site. A similar result has been reported for the TATA element in the Ac*M*NPV 35K gene (10). However, a different result was observed when the proximal start sites were mutated in the baculovirus 39K gene. In plasmids p39LS-H and -I, transcription initiation from the proximal sites was abolished.

The proximal transcripts initiate at a CAGT motif. Similar sequence elements are involved in transcription initiation in several systems and may function by both TATA-independent and TATA-dependent mechanisms, depending on the promoter. The sequence (CTCCANTCT) functions as an initiator element in mammalian cells (35, 36). Initiator-mediated transcription is TATA independent, although it is stimulated by a TATA element, and may interact with TFII-D (36). The sequence TCAGTT has been shown to function as an initiator for the gypsy retrotransposon (22). The gypsy promoter lacks a TATA element and requires a downstream sequence in addition to the CAGT motif. A similar sequence (ATCA[G/T]TY) is also found at the initiation sites of several *Drosophila* and *Bombyx* genes (21). These insect genes contain TATA boxes, and the distance from the transcription start site (A of the CAGT motif) to the first T of the TATA box is conserved at 28 to 31 nucleotides, similar to what was found for the AcNPV 39K gene reported here. *Drosophila* heat shock genes also initiate at a conserved CAGTT motif, which is located 32 to 33 nucleotides downstream of a TATA box (21). Mutational analysis of the *Drosophila* sequence indicates that it is essential for transcription initiation.

Alteration of the CAGT motif in the 39K promoter abolished transcription from the proximal sites. This finding suggests that the CAGT motif functions as an initiator element, similar to the initiator motifs described above. The distal transcripts do not initiate at a CAGT motif and were unaffected by alterations in CAGT or at the distal start site. A possible explanation for this observation is that efficient binding of TFIID to the proximal TATA box requires interactions at the initiation site, whereas binding of TFIID to the distal site is stable in the absence of downstream interactions. This model is similar to that previously proposed for a human gene promoter which requires a downstream initiator for efficient binding of TFIID to the TATA box (28). The exact mechanism that distinguishes the function of the two 39K TATA elements is unknown. The TATA elements are similar in sequence, and both match the consensus TATA sequence (TATAAA and TATATA). On the basis of binding affinities of yeast TFIID to consensus TATA elements (18), we would predict that TFIID should bind both sequences with equal affinities. It is possible that the sequences surrounding the TATA sequences could affect the binding affinities, as it has been demonstrated that the yeast TFIID footprint is larger than the 6-bp consensus sequence (18). Another possibility is that the difference between the two TATA elements is related to the fact that the proximal TATA element overlaps the baculovirus late promoter sequence (TAAG). Although considerable evidence suggests that late genes are transcribed by a virus-specific polymerase, it is possible that host transcription factors interact with this sequence.

The exact role of the CAGT motif in the baculovirus life cycle is unknown. However, the similarity of the CAGT with known initiator motifs (21, 22, 35), and the results presented here, suggests that the CAGT motif functions directly in RNA initiation by binding a specific transcription factor. Alternatively, it is possible that the CAGT motif affects the stability of these transcripts. Because transcription initiates at the A residue of this sequence, the nucleotides AGT would be found at the 5′ end of the early transcripts initiating at this sequence.

The data presented here stress the importance of transcription mapping in addition to CAT assays. Because the two TATA elements controlled transcription separately, the CAT assay data alone did not accurately reflect the effects of mutations. For example, CAT activity in p39LS-H was equivalent to wild-type activity, apparently indicating that the mutated CAGT motif was not involved in transcription. However, examination of the transcription pattern indicated

that this mutation abolished transcripts initiating at the CAGT motif. This decrease in proximal transcription was offset by an increase in transcription from the distal site, resulting in high levels of CAT activity (Fig. 2).

We have previously shown that the 39K promoter consists of tandem early and late elements (15). A similar promoter structure has also been described for the gp64 gene and the IE0 gene (1, 23). This arrangement should allow for expression of these proteins both early and late in infection, although it is not known whether these proteins are essential during both phases. The gp64 gene encodes the major membrane glycoprotein. Viruses generally express structural genes during the late phase, and the role, if any, of gp64 during the early phase is unknown. We have recently shown that the protein encoded by the 39K gene is associated with the nuclear matrix (14), although the exact function of this protein remains to be elucidated. Although we have also shown that 39K mRNA is abundant during both the early and late phases (15), we do not know whether the protein product is expressed throughout infection. Expression of 39K during the early phase is further regulated by dual TATA elements. The fact that these TATA elements are functionally distinct may allow for an additional level of control during the early phases. The multiple levels of 39K regulation suggest that the protein plays an important role in virus infection.

Six early genes of AcNPV that contain CAGT motifs at the initiation site have been described (1, 6, 8, 14, 17, 25). An equivalent number of other early genes that lack this initiator motif have been sequenced (9, 27, 29). There is no obvious common factor that links these genes, with respect to either temporal expression or function of the gene products. Three of the genes that contain CAGT are immediate-early regulatory genes; IEN and IE0 are expressed transiently (5, 8), while IE1 is expressed throughout infection (17). Two other genes containing CAGT, 39K and gp64, were discussed above. They are similar to each other with respect to temporal expression but different from the immediate-early regulatory genes. Another CAGT-containing gene is PE-38, a transiently expressed immediate-early gene of unknown function (25). Further analysis of the role of CAGT in initiation of baculovirus transcription will require identification of proteins that interact with this element.

The 39K gene contains a minicistron upstream of the p31 open reading frame, suggesting that expression of this protein may also be regulated at the level of translation. One of the linker insertion plasmids, p39LS-J, contained mutations that altered the ATG codon of the minicistron. The levels of CAT induced in this plasmid were equivalent to the levels induced by p39LS-WT. Primer extension results indicated that this plasmid was transcribed at wild-type levels. These results suggest that the presence of a functional minicistron does not affect expression of the 39K gene, at least not in this transient assay. Minicistrons have also been identified in the IE0 gene of Ac*M*NPV and the gp64 genes of Ac*M*NPV and a related baculovirus, *Orygia pseudotsugata* multicapsid nuclear polyhedrosis virus (1, 40). The presence of minicistrons in three Ac*M*NPV genes and the conservation of this structure in gp64 genes in two different baculoviruses argues that minicistrons may play a role in baculovirus-infected cells, even though an effect was not detected in this analysis.

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