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The CAGT Motif Functions as an Initiator Element during Early Transcription of the Baculovirus Transregulator *ie-1*

STEVEN S. PULLEN AND PAUL D. FRIESEN*

Institute for Molecular Virology and Department of Biochemistry, Graduate School and College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

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The highly conserved tetranucleotide CAGT is located at the RNA start site of the transregulator gene *ie-1* of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV). The presence of this motif within numerous baculovirus early promoters and its similarity to transcriptional initiators suggested a fundamental role in viral transcription regulation. To determine the function of the CAGT motif, site-specific mutations were introduced within the *ie-1* promoter fused to a reporter gene within Ac*M*NPV recombinants. In previous studies, deletion of the CAGT motif (nucleotides −1 to +3) and the adjacent downstream activating region (nucleotides +11 to +24) abolished *ie-1* transcription. Here, we show that nucleotide replacements within the CAGT motif reduced steady-state levels of *ie-1* RNAs from the proper start site (+1), both early and late in infection. These CAGT mutations caused comparable reductions in the yield of *ie-1* runoff RNAs from in vitro transcription reactions using nuclear extracts from Ac*M*NPV-infected cells; the CA dinucleotide was most sensitive to substitution. Thus, the CAGT motif affects the rate of *ie-1* transcription. Deletions upstream and downstream from the *ie-1* RNA start site demonstrated that nucleotides −6 to +11 encompassing the CAGT motif were sufficient for proper transcription in a TATA-independent manner. Nonetheless, additional regulatory elements, which included the *ie-1* TATA element, the *ie-1* downstream activating region, and a heterologous upstream activating region, stimulated transcription from the motif. Thus, by all criteria examined, the *ie-1* CAGT motif functions as a transcriptional initiator by its capacity to determine the position of the RNA start site and to regulate the rate of transcription. These findings suggest that by stimulating early transcription through the recruitment of host factors, the CAGT initiator accelerates expression of viral genes, such as *ie-1*, that are critical to establishing a productive infection.

Productive infection by *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) requires the proper expression of early and late genes that are dispersed throughout its circular, 134-kbp DNA genome. Since early viral genes regulate late replicative processes as well as the host insect's response to infection, these genes can function as determinants of Ac*M*NPV host range (for baculovirus reviews, see references 4 and 38). Current evidence indicates that baculovirus early gene expression is controlled primarily at the level of transcription. Early promoters are responsive to host RNA polymerase II and are composed of multiple *cis*-acting regulatory components, including the TATA element and distinct motifs located within the upstream activating region (UAR) or the 5′ noncoding leader region. These *cis*-acting elements regulate early promoter activity by recruiting host- and virus-encoded factors that are involved in potentiating transcription (2, 7, 13, 17, 27, 28, 32, 35, 37, 40, 45, 49).

The tetranucleotide CAGT is positioned at or near the RNA start sites of numerous baculovirus genes (3, 7, 12, 18, 19, 26, 33, 49, 50, 54). The core motif (CAGT) not only resembles the RNA start sites of many insect genes (21) but also matches the proposed consensus (A/C/T)CA(G/T)T for arthropod transcriptional initiator elements (10). Initiator elements overlap the RNA start site of RNA polymerase II promoters, contribute to basal promoter activity, and position the RNA start site, even in the absence of a TATA element (for a review, see reference 53). Initiators have been identified at the transcriptional start sites of vertebrate genes and several genes of mammalian DNA viruses, including adenovirus, simian virus 40, and polyomavirus (1, 9, 47, 55). Although the striking conservation of the CAGT motif at the RNA start of baculovirus genes suggests that it plays a central role in transcriptional regulation, the function and significance of the motif for baculovirus replication are unknown.

A CAGT motif (nucleotides −1 to +3) is located at the RNA start site (+1) of the Ac*M*NPV transregulator gene *ie-1* (see Fig. 1). The *ie-1* protein (IE1) is a multifunctional transregulator that affects early virus promoters in transfection assays (6, 17, 19, 31, 37, 39, 40) and has been implicated as one of six viral proteins essential for DNA replication in transient replication assays (29). During infection, IE1 accelerates viral replication, as indicated by a temperature-sensitive mutant (43). Transcription of *ie-1* begins immediately after infection; RNAs initiate from the CA dinucleotide within the CAGT motif and are present throughout infection (12, 19, 30, 40). The *ie-1* promoter contains a functional TATA element, which is located 30 bp upstream from the RNA start site, and a downstream activating region (DAR), which extends from nucleotides +11 to +24 (40). During infection, the DAR maintains maximal steady-state *ie-1* RNA levels by providing a 10-fold stimulation of the rate of *ie-1* transcription. However, simultaneous deletion of the CAGT motif and the DAR abolished *ie-1* RNAs (40). This finding suggested that the CAGT motif, possibly in combination with the DAR, is critical for *ie-1* transcription.

To determine the function of the CAGT motif during *ie-1* expression, we analyzed mutated *ie-1* promoters that were fused to the chloramphenicol acetyltransferase (CAT) reporter gene by using Ac*M*NPV recombinants and in vitro transcrip-

* Corresponding author. Mailing address: Institute for Molecular Virology, Bock Laboratories, University of Wisconsin-Madison, 1525 Linden Dr., Madison, WI 53706-1596. Phone: (608) 262-7774. Fax: (608) 262-7414. Electronic mail address: PFriesen@psl.wisc.edu.

tion assays. We report here that mutations of the CAGT motif reduced the rate of *ie-1* transcription early in infection. Moreover, *ie-1* sequences from −6 to +11, containing the CAGT motif, were sufficient for proper transcription initiation. The *ie-1* TATA element and DAR, as well as the heterologous UAR from the Ac*M*NPV *p35* gene, each stimulated transcription from the motif. Thus, by definition, the CAGT motif represents a transcriptional initiator element for *ie-1*. This is the first demonstration that a CAGT motif functions as an initiator during baculovirus infection. Our data suggest that identical motifs at the RNA start sites of other early baculovirus genes function similarly.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* IPLB-SF21 (SF21) (51) cells were propagated in TC100 growth medium (GIBCO Laboratories) supplemented with 2.6 mg of tryptose broth per ml and 10% heat-inactivated fetal bovine serum. For infection, cell monolayers were inoculated with virus at a multiplicity of infection of 20 PFU per cell. After a 1-h adsorption period, the cells were washed with TC100, covered with growth medium, and incubated at 27°C. Time zero was defined as the point at which virus was added. Standard gene replacement methods were used to construct Ac*M*NPV (L-1 strain) recombinants with pEVocc$^+$/PA-based transfer vectors as previously described (13). In some cases, purified DNA from recombinant virus wt/lacZ (20), linearized with *Bsu* 36I within the *Escherichia coli lacZ* gene, was used as described by Kitts et al. (25). Recombinant viruses were plaque purified and verified for proper insertion of desired sequences by restriction mapping of purified viral DNA. Viruses vIE1-FCAT and v35K-CAT have been described previously (13, 40).

Plasmids. The *Xho*I-*Xba*I fragment containing the *ie-1* promoter-CAT fusion of each CAT plasmid described below was inserted into the corresponding sites of transfer vector pEVocc$^+$/PA as described previously (40). Site-directed mutations and inserted fragments were verified by dideoxy-chain termination sequencing (46) of all pEVocc$^+$/PA-based plasmids. Plasmid pIE1-FCAT contains the *ie-1* promoter and 8 codons of the *ie-1* open reading frame (ORF) (nucleotides −161 to +74, relative to the RNA start site at +1) fused in frame to the CAT gene (40).

(i) CAGT mutations. Plasmid pIE1$_{Bg3}$-FCAT contains a *Bgl*II site centered at position −10 of pIE1-FCAT (40). The oligonucleotides 5′-GTCAACTTGCAACTGTGACAATATCCAACATGAACG-3′, 5′-GTCAACTTGCAACTCACACAATATCCAACATGAACG-3′, 5′-GTCAACTTGCAACGTAAACAATATCCAACATGAACG-3′, 5′-GTCAACTTGCACATGAAACAATATCCAACATGAACG-3′, and 5′-GTCAACTTGCAGTACAAACAATATCCAACATGAACG-3′ were used to make the indicated nucleotide changes (underlined) by site-directed mutagenesis (34) near the *ie-1* RNA start site of pIE1$_{Bg3}$-FCAT; nucleotides complementary to the CAGT motif are overlined. The resulting plasmids were designated CAGT(−3,−2), CAGT(−3,−1), CAGT(−1,+1), CAGT (+2,+3), and CAGT(−1/+3), respectively. In each case, the *Bgl*II site of pIE1$_{Bg3}$-FCAT was eliminated and wild-type *ie-1* sequences were restored.

(ii) Hybrid *ie-1* promoters. Plasmid IE1-CAGT was generated by *Bgl*II and *Sal*I digestion of pIE1$_{Bg3}$-FCAT, end repair with the Klenow fragment, and intramolecular ligation; the *Sal*I site was located upstream from the *ie-1* promoter fragment within vector sequences. A 204-bp *Xho*I-*Bgl*II fragment from p(Δ3′-32/Δ5′-30)35K-CAT (13), containing the *p35* UAR but not the TATA element (nucleotides −162 to −32, relative to the +1 RNA start), was inserted at *Xho*I and *Bgl*II sites of pIE1$_{Bg3}$-FCAT to generate pIE1-CAGT/35K$_{UAR}$(+1). A 495-bp end-repaired *Bam*HI fragment from p*hr*5^B (45), containing the Ac*M*NPV *hr*5 enhancer, was inserted into pIE1$_{Bg3}$-FCAT at end-repaired *Bgl*II and *Xho*I sites to generate pIE1-CAGT/hr5$^+$ or pIE1-CAGT/hr5$^-$, in which + and − designate the genome and opposite orientation of *hr*5, respectively. The 898-bp *Bgl*II-*Xba*I fragment from pIE1-CAGT/35K$_{UAR}$(+1), possessing *ie-1* nucleotides −6 to +74 fused to the CAT gene, was inserted into the *Bam*HI and *Xba*I sites of the pBluescript (KS$^+$) vector (Stratagene) to generate pBS^{MCS}/IE1-CAGT. A 204-bp *Xho*I-*Bgl*II (end repaired) fragment from pIE1-CAGT/35K$_{UAR}$(+1), containing the *p35* UAR, was inserted into pBS^{MCS}/IE1-CAGT, which was digested either with *Sma*I, *Eco*RI, or *Eco*RV and then with *Xho*I to generate pIE1-CAGT/35K$_{UAR}$(+9), pIE1-CAGT/35K$_{UAR}$(+23), and pIE1-CAGT/35K$_{UAR}$(+27), respectively; the *Eco*RI site was end repaired. Likewise, the 223-bp *Kpn*I-*Bgl*II (end-repaired) fragment from pIE1-CAGT/35K$_{UAR}$(+1), containing the *p35* UAR, was inserted into pBS^{MCS}/IE1-CAGT, which was digested with either *Hin*dIII or *Sal*I and then with *Kpn*I to generate pIE1-CAGT/35K$_{UAR}$(+35) and pIE1-CAGT/35K$_{UAR}$(+50), respectively; the *Hin*dIII and *Sal*I sites were end repaired. pIE1-CAGT/35K$_{UAR}$(+27) was digested with *Xba*I and was partially digested with *Hin*cII to isolate the 3,153-bp vector fragment linearized at the *Hin*cII site at *ie-1* nucleotide +11. Subsequently, an 851-bp *Hin*cII-*Xba*I fragment from pIE1(Δ+36/+74)-FCAT/PA, an 839-bp *Hin*cII-*Xba*I fragment from pIE1(Δ+24/+74)-FCAT/PA, or an 809-bp *Hin*dIII (end-repaired)-*Xba*I fragment from pIE1$_{UAR}$35K$_{BAS}$-CAT (40) was inserted to generate plasmids pIE1-CAGT(Δ+30/+74), pIE1-CAGT(Δ+17/+74), and pIE1-CAGT(Δ+11/+74), respectively.

(iii) *39K* and *gp64* promoters. The *39K* promoter (18) from nucleotides −245 to +132, relative to the proximal early RNA start site (+1), was amplified from L-1 Ac*M*NPV DNA by PCR methods and was inserted into pBluescript (KS+) at *Xho*I and *Hin*dIII sites to generate plasmid 39K-prm. Site-directed mutagenesis of plasmid 39K-prm with oligonucleotide 5′-GGCCTAAATTAGCTCTAGATCTTTCTGGATCCCGCTCAAACGACACGATG-3′ replaced the proximal and distal TATA elements with *Bgl*II and *Bam*HI sites (underlined), respectively, to generate plasmid 39K-TATAs$^-$. Digestion of plasmid 39K-TATAs$^-$ with *Bgl*II and *Dra*I (vector-encoded site), end repair, and intramolecular ligation generated plasmid 39K-CAGT. Digestion of p39K-TATAs$^-$ with *Bam*HI, end repair, and intramolecular ligation generated plasmid 39K$_{UAR}$. The *gp64* promoter (54) from nucleotides −187 to +42, relative to the early RNA start site (+1), was amplified from L-1 Ac*M*NPV DNA by PCR methods and was cloned into pBluescript (KS+) at *Bam*HI and *Eco*RI sites to generate plasmid gp64-prm. Site-directed mutagenesis of plasmid gp64-prm with oligonucleotide 5′-GCCAGATAACGGTCGGGAGATCTAGATGCCTCAATGCTAC-3′ replaced the TATA element with a *Bgl*II site (underlined) to generate plasmid gp64-TATA$^-$. Digestion of plasmid gp64-TATA$^-$ with *Bgl*II and *Not*I (vector-encoded site), end repair, and intramolecular ligation generated plasmid gp64-CAGT. Digestion of plasmid gp64-TATA$^-$ with *Bgl*II and *Eco*RI, end repair, and intramolecular ligation generated plasmid gp64$_{UAR}$. Site-directed mutations and inserted fragments were verified by dideoxy-chain termination sequencing (46).

Primer extension analysis of RNA. Total RNA was isolated from infected cells by using the guanidine isothiocyanate-cesium chloride method (11). Primer extension quantitation assays were performed as previously described (13, 37) with excess 5′-end-labeled oligonucleotide primers complementary to CAT (nucleotides +15 to +53 relative to the ATG initiation codon) and *p35* (nucleotides +53 to +90). Extension products were subjected to electrophoresis on 6% polyacrylamide–8 M urea–TBE (100 mM Tris-borate [pH 8.3], 2 mM EDTA) gels and to autoradiography. Dideoxy-chain termination sequencing ladders were generated with ^{35}S-dATP (Amersham) and the CAT-specific primer annealed to pIE1-FCAT/PA DNA. Radiolabeled primer extension products were quantitated by using a Betagen Betascope 603 blot analyzer.

In vitro transcription using SF21 nuclear extracts. Nuclear extracts were prepared from uninfected or Ac*M*NPV-infected SF21 cells as described previously (40). In vitro transcription reactions using [α-^{32}P]CTP were also conducted as described elsewhere (40). Templates containing the *39K* or *gp64* promoters were linearized at the *Pvu*II site, which was located 324 or 319 nucleotides downstream of each RNA start site, respectively, whereas templates containing the CAT gene were linearized at the *Eco*RI site. Quantitation was performed by using a Betagen Betascope 603 blot analyzer.

Image processing. Autoradiograms were scanned at a resolution of 300 dpi by using a Microtek Scanmaker III equipped with a transparency adapter. The resulting files were printed from Adobe Photoshop 2.5 by using a Tektronix Phaser IISDX dye sublimation printer.

RESULTS

The CAGT motif is required for maximal early *ie-1* transcription. The RNA start site of *ie-1* (Fig. 1) is composed of the tetranucleotide CAGT found at or near the same position relative to the TATA element of many baculovirus early genes. We previously observed that deletion of *ie-1* promoter sequences that included the CAGT motif abolished transcription early and late in infection (40). This finding suggested that proper *ie-1* expression required the CAGT motif. Thus, to determine the function of the CAGT motif in directing *ie-1* transcription, nucleotide replacements within the motif were constructed (Fig. 2A). These mutations were introduced into the *ie-1* promoter (extending from nucleotides −161 to +74) that was fused to the CAT reporter gene (see Fig. 3A) and then inserted at the polyhedrin locus of Ac*M*NPV recombinants. The *ie-1* promoter exhibits proper (wild-type) temporal regulation when placed at this genomic location (40). The level of early (4-h) IE1-FCAT RNA from each CAGT mutant was determined by primer extension assays in which RNA from the Ac*M*NPV *p35* gene served as an internal control for total RNA levels. Hereafter, IE1-FCAT RNA refers to transcripts extending from the *ie-1* start site through the CAT gene.

All CAGT mutations tested reduced steady-state levels of IE1-FCAT transcripts early in infection (Fig. 2B). Substitutions of nucleotides −1 to +1 [CAGT(−1,+1)] or −1 to +3

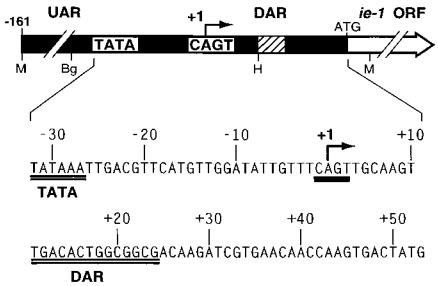

FIG. 1. Structural organization of the *ie-1* promoter. The promoter of the AcMNPV *ie-1* gene (94.5 to 96.2 map units) includes the UAR, TATA element, DAR, and the CAGT motif which contains the RNA (+1) start site (small arrow). Each regulatory element is located upstream from the ATG initiation codon (+51) of the *ie-1* ORF (open arrow). The nucleotide sequence of the *ie-1* promoter from nucleotides −32 to +53 is shown below; sequences consisting of the CAGT motif (solid bar), the TATA element (doubly underlined), and the DAR (doubly underlined) are indicated. Restriction site abbreviations: Bg, *Bgl*I; M, *Mlu*I; H, *Hin*cII.

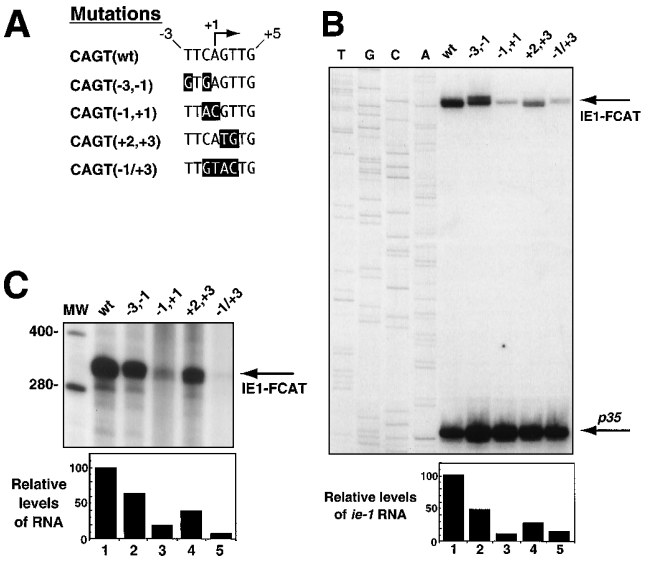

FIG. 2. Effect of CAGT motif mutations on early *ie-1* transcription. (A) RNA start site mutations. The nucleotide sequence of the wild-type (wt) CAGT motif and mutations thereof are shown; each mutant is designated according to the position of nucleotide replacements (outlined in black). The mutations were introduced into plasmid pIE1-FCAT/PA, which was used to construct AcMNPV recombinants in which *ie-1* sequences (−161 to +74) were fused in frame with the CAT gene and inserted at the polyhedrin locus. (B) Primer extension of early IE1-FCAT RNA. Total RNA isolated 4 h after infection with recombinant viruses (multiplicity of infection of 20) containing the indicated mutations was quantitated by primer extension using a 5′-end-labeled CAT primer; as an internal control, each RNA sample was simultaneously annealed to a 5′-end-labeled primer complementary to *p35*. The extension products generated from equivalent amounts (14 μg) of RNA were subjected to polyacrylamide gel electrophoresis and autoradiography; arrows denote IE1-FCAT- and *p35*-specific products. The histogram at the bottom shows the level of IE1-FCAT RNA normalized to that of *p35*. A dideoxy-chain sequencing ladder was generated with pIE1-FCAT/PA and the same CAT primer to locate the *ie-1* RNA start site; an identical sequencing ladder is used in subsequent figures. (C) In vitro transcription. SF21 nuclear extracts prepared 4 h after infection were programmed with DNA templates (500 ng) derived from pIE1-FCAT/PA plasmids containing the indicated CAGT mutations; templates were linearized at the *Eco*RI site within the CAT gene. ^{32}P-labeled runoff products were subjected to polyacrylamide gel electrophoresis and autoradiography; the 333-nucleotide IE1-FCAT runoff transcript is denoted by the arrow. The sizes of radiolabeled RNAs used as molecular weight (MW) markers are indicated in nucleotides. The yields of IE1-FCAT transcripts were measured by using a betascope and were plotted as a histogram (below).

[CAGT(−1/+3)] caused a 10-fold decrease in steady-state RNA levels, which was the most dramatic of the reductions observed. In the case of both mutants, the purine/pyrimidine content of the CAGT motif was maintained. Inversion of the G and T at positions +2 and +3 [CAGT(+2,+3)] reduced steady-state levels of transcripts fourfold, whereas replacement of nucleotides −3 and −1 [CAGT(−3,−1)] reduced RNA levels twofold. As judged by the sizes of the extension products (Fig. 2B), transcripts initiated at or near the wild-type +1 position for each mutation.

To determine if the CAGT motif regulates the level of early *ie-1* RNAs by affecting the rate of *ie-1* transcription, the activities of DNA templates containing each CAGT mutation were tested by in vitro transcription reactions using nuclear extracts prepared from cells early after infection (Fig. 2C). As shown previously (40), in vitro transcripts from the *ie-1* promoter initiate from the proper start site (+1) within the CAGT motif. Substitution of the four core nucleotides −1 to +3 [CAGT(−1/+3)] reduced the yield of runoff transcripts by at least tenfold compared with that of the wild-type motif (Fig. 2C, lanes 1 and 5). Substitution of nucleotides −1 and +1 [CAGT(−1/+1)] and inversion of the G and T [CAGT(+2,+3)] reduced runoff transcription fivefold and threefold, respectively (Fig. 2C, lanes 3 and 4). The smallest reduction (twofold) was caused by replacement of nucleotides −3 and −1 [CAGT(−3,−1)]. In vitro transcription using nuclear extracts from uninfected cells showed similar reductions when CAGT mutations were compared with the wild-type motif (data not shown). Thus, the effect of each CAGT mutation on the in vitro activity of the *ie-1* promoter closely paralleled the reduction in steady-state levels of *ie-1* RNA early after infection with recombinant viruses containing the same mutations (Fig. 2B). This finding indicated that CAGT mutations altered the steady-state levels of viral *ie-1* RNA by affecting the rate of early transcription rather than RNA stability. Owing to its capacity to stimulate transcription, the CAGT motif is therefore necessary for maximal *ie-1* RNA levels early in infection. These assays also confirmed that *ie-1* transcription is accurately reproduced by in vitro transcription reactions (40).

The *ie-1* promoter is active in the absence of its TATA element. Because of its similarity to the consensus sequence compiled for initiator elements (10, 23), the *ie-1* CAGT motif may function as a transcriptional initiator element. An initiator is a DNA sequence that overlaps the transcription start site and is sufficient for (i) determining the position of the start site in a promoter that lacks a TATA element and (ii) contributing to the strength of a promoter that contains a TATA element (23, 47). To assess the activity of the *ie-1* CAGT motif in the absence of a TATA element, recombinant virus vIE1-CAGT was constructed by deleting all *ie-1* sequences upstream of nucleotide −6, including the TATA element (Fig. 3A). Primer extension analysis of RNA isolated early (4 h) after infection with vIE1-CAGT revealed that *ie-1* promoter sequences from −6 to +74 were sufficient to direct transcription from the CAGT motif (Fig. 3B, lane 5). Although the steady-state level of IE1-FCAT RNA from the TATA-less vIE1-CAGT promoter was ~50-fold lower than that of vIE1-FCAT containing the full-length *ie-1* promoter (Fig. 3B, lane 1), transcription initiated from the proper (+1) start site. Since the *ie-1* UAR (nucleotides −161 to −34) contributes little, if any, stimulation of *ie-1* transcription (40), the reduction in levels of IE1-FCAT RNA from those of vIE1-CAGT was due to loss of the TATA element.

In a confirmation of the activity of the TATA-less *ie-1* promoter, in vitro transcription of DNA templates lacking the *ie-1* TATA element (IE1-CAGT) yielded runoff RNAs with a mo-

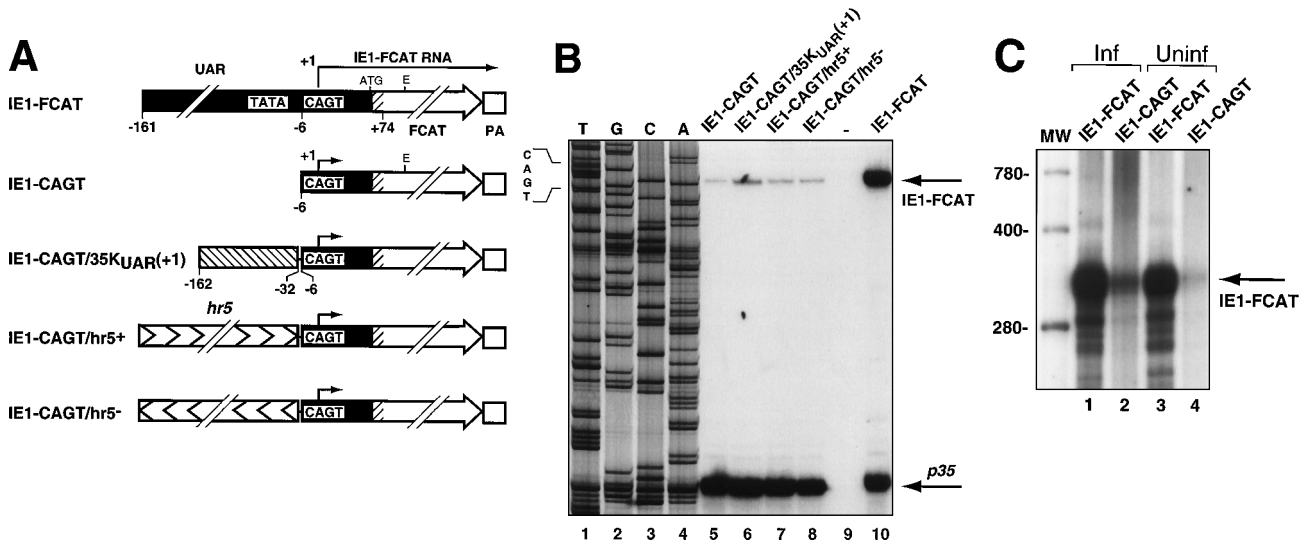

FIG. 3. *ie-1* promoter activity in the absence of a TATA element. (A) Promoter organization. The IE1-FCAT RNA (arrow) extends downstream from the *ie-1* start site (+1) within the CAGT motif of each plasmid; the first eight codons of *ie-1* (striped box), including the ATG, were spliced in frame with the CAT gene (FCAT), which is followed by a polyadenylation signal (PA). IE1-FCAT contains the wild-type *ie-1* promoter from nucleotides −161 to +74, whereas IE1-CAGT contains nucleotides −6 to +74. IE1-CAGT/35K$_{UAR}$(+1) contains the *p35* UAR (*p35* nucleotides −162 to −32). IE1-CAGT/*hr5*$^{+}$ and IE1-CAGT/*hr5*$^{-}$ contain the Ac*M*NPV *hr5* enhancer (484 bp) in opposite orientations. Recombinant viruses were constructed by using each promoter plasmid as described in the legend to Fig. 2. E, *Eco*RI. (B) Primer extension quantitation of early RNA. Equivalent amounts (11 μg) of total RNA isolated 4 h after infection with the indicated recombinant viruses were primer extended by using the 5′-end-labeled CAT primer as described in the legend to Fig. 2. To preserve sample purity, lanes 8 and 10 were separated by a blank (−) lane (lane 9). (C) In vitro transcription. Nuclear extracts from either Ac*M*NPV infected (Inf) or uninfected (Uninf) SF21 cells were programmed with *Eco*RI-linearized DNA templates (750 ng) from pIE1-FCAT/PA or pIE1-CAGT/PA; ^{32}P-labeled runoff transcripts were analyzed as described in the legend to Fig. 2. Proper initiation from both plasmid templates yielded runoff transcripts of 333 nucleotides (arrow). RNA molecular weight (MW) markers are indicated at the left.

bility consistent with that predicted (333 nucleotides) (Fig. 3C, lanes 2 and 4). TATA-less *ie-1* transcription was significantly lower than that from the TATA-containing promoter (IE1-FCAT) (Fig. 3C, lanes 1 and 3), which is consistent with the reduced level of TATA-less transcription from virus vIE1-CAGT (Fig. 3B). Since the TATA-less *ie-1* promoter was active in nuclear extracts from uninfected cells, producing runoff RNAs with identical sizes (Fig. 3C), host factors were sufficient for TATA-independent transcription. This finding was consistent with the response of initiator elements from other viruses to host transcription factors (1, 5, 47, 55). Taken together, our data indicated that *ie-1* transcription initiated properly from the CAGT motif in a TATA-independent manner and that the TATA element, when present, stimulated the rate of transcription.

TATA-less *ie-1* promoter activity is stimulated by a heterologous UAR. A common property of initiators is stimulation by upstream activating elements in the absence of a TATA element (5, 8, 36, 47). To determine whether transcription from the TATA-less *ie-1* promoter can be augmented by heterologous upstream activating elements, we measured the effect of placing the UAR of the *p35* promoter or the Ac*M*NPV *hr5* enhancer immediately upstream of the *ie-1* promoter that extended from nucleotides −6 to +74 (Fig. 3A); the UAR and *hr5* lack a TATA element. Our previous studies using similar Ac*M*NPV recombinants showed that the *p35* UAR and *hr5* increased early transcription from the TATA-containing *ie-1* promoter (nucleotides −34 to +74) by 11-fold and 4-fold, respectively, whereas the *ie-1* UAR had little effect (40). In the present study, the *p35* UAR also increased transcription from the TATA-less *ie-1* promoter (Fig. 3); the steady-state level of early IE1-FCAT RNAs of recombinant vIE1-CAGT/35K$_{UAR}$(+1) was 40% higher than that of vIE1-CAGT (Fig. 3B, lanes 5 and 6). In contrast, *hr5* had no detectable effect in either orientation (vIE1-CAGT/*hr5*$^{+}$ or vIE1-CAGT/*hr5*$^{-}$) (lanes 7 and 8).

To test the position dependency of *p35* UAR stimulation, the distance between the UAR and the TATA-less *ie-1* promoter was altered by inserting pBluescript sequences (Fig. 4A). As the distance between the UAR and RNA start site was increased, the level of early (4 h) steady-state IE1-FCAT RNA also increased (Fig. 4B). Optimum stimulation of *ie-1* promoter activity was observed when the *p35* UAR was located 27 bp from the RNA start site, which is the same distance between the UAR and RNA start site for *p35* (13); IE1-FCAT RNA levels of vIE1-CAGT/35K$_{UAR}$(+27) were 2.5-fold higher than those of vIE1-CAGT/35K$_{UAR}$(+1) (Fig. 4B, lanes 1 and 4). Increasing the distance beyond 27 bp reduced UAR stimulation, as shown by viruses vIE1-CAGT/35K$_{UAR}$(+35) and vIE1-CAGT/35K$_{UAR}$(+50). Thus, promoter stimulation by the pBluescript spacer DNA was ruled out. Overall, the *p35* UAR provided a 3.5-fold increase in TATA-less promoter activity over that of vIE1-CAGT (Fig. 3). A similar position dependence has been observed for stimulation of the initiator element of the terminal deoxynucleotidyltransferase gene by transcription activator Sp1 (47).

Defining sequences required for TATA-less *ie-1* promoter activity. Deletions from the 3′ side of the *ie-1* promoter from +74 were generated to determine the role of sequences within the 5′ noncoding leader region in TATA-less transcription (Fig. 5A). Each deletion was analyzed in the context of the TATA-less *ie-1* promoter (−6 to +74) in which the *p35* UAR was inserted 27 bp upstream for optimal transcription activation during infection with recombinant viruses. Compared with the full-length *ie-1* leader region [vIE1-CAGT/35K$_{UAR}$(+27)] (Fig. 5B, lane 5), removal of nucleotides +30 to +74 increased steady-state levels of early RNA [vIE1-CAGT (Δ+30/+74)] (lane 6), whereas deletion of +17 to +74 had little effect

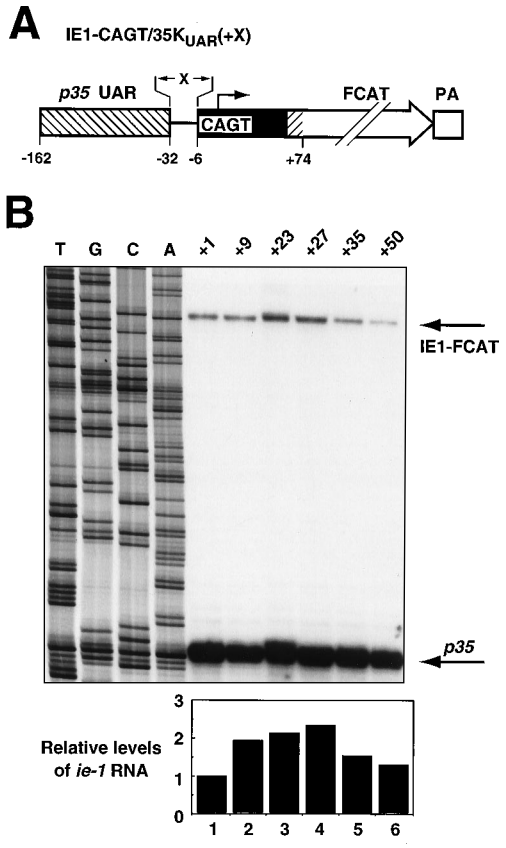

FIG. 4. Position dependence of *p35* UAR stimulation of TATA-less *ie-1* promoter activity. (A) Organization of IE1-CAGT/35K$_{UAR}$(+X) promoters. The distance between the UAR (*p35* nucleotides −162 to −32) and the TATA-less *ie-1* promoter (nucleotides −6 to +74) was increased from 1 to 50 bp by inserting pBluescript sequences. Each promoter, designated by the size of the insertion in nucleotides (+X), was fused to the CAT gene and used to construct recombinant viruses. (B) Primer extension quantitation of early RNA. Equivalent amounts (11 μg) of total RNA isolated 4 h after infection with the indicated recombinant virus were primer extended by using the 5′-end-labeled CAT primer as described in the legend to Fig. 2. The histogram (below) shows the levels of IE1-FCAT RNAs normalized to that of *p35*.

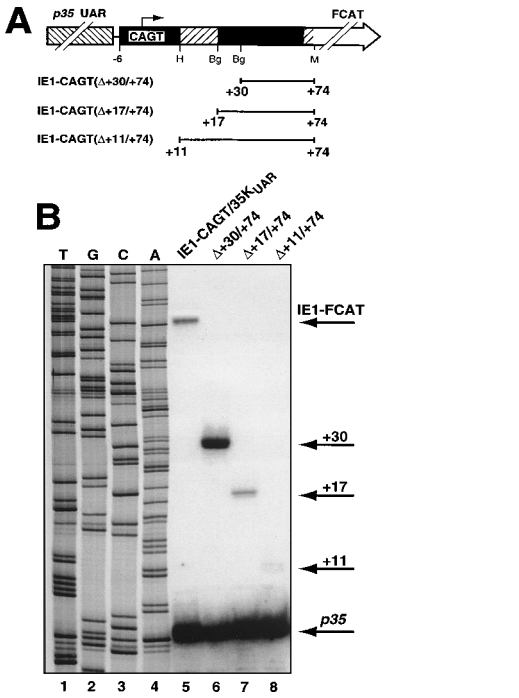

FIG. 5. Defining minimal TATA-less *ie-1* promoter sequences. (A) Deletion boundaries within the *ie-1* 5′ leader region. The nucleotides removed for each of the IE1-CAGT/35K$_{UAR}$(+27) promoters listed at the left are depicted by a solid line below the *ie-1* noncoding region and the ORF; deletion endpoints which represent *ie-1*-specific nucleotides are indicated. *Bgl*II sites used to generate internal deletions were introduced by site-directed mutagenesis. Bg, *Bgl*II; H, *Hin*cII; M, *Mlu*I. (B) Primer extension quantitation of early RNA. Equivalent amounts (14 μg) of total RNA isolated 4 h after infection with the indicated recombinant viruses were primer extended by using the 5′-end-labeled CAT primer as described in the legend to Fig. 2; arrows indicate the extension products specific for *p35* and each IE1-CAGT promoter.

[vIE1-CAGT (Δ+17/+74)] (lane 7). In contrast, removal of nucleotides +11 to +74, which included the *ie-1* DAR, decreased early RNA levels [vIE1-CAGT(Δ+11/+74)] (lane 8). Nevertheless, the remaining *ie-1* sequences (−6 to +11) were sufficient to direct early transcription from the proper site within the CAGT motif. Therefore, nucleotides −6 to +11 contain the minimal sequences required for TATA-less *ie-1* promoter activity early during infection. Moreover, since the DAR affects the rate of *ie-1* transcription rather than RNA stability (40), this downstream element must function by stimulating transcription from the CAGT motif.

Transcriptional activities of other Ac*M*NPV CAGT motifs. The 39K gene (*39K*) and the major glycoprotein gene *gp64* both contain a conserved CAGT motif at their RNA start sites (3, 18, 54). To test whether each motif was also sufficient for transcription, we compared the in vitro activities of both promoters with and without their TATA elements to that of the TATA-less *ie-1* promoter (Fig. 6A). The full-length *39K* and *gp64* promoters were active in nuclear extracts prepared from infected SF21 cells, since each produced runoff RNAs of the predicted sizes (Fig. 6B, lanes 3 and 7). As shown previously, in vitro transcripts from either promoter initiate from the CAGT motif (2, 16). Nucleotide replacements that eliminated the TATA elements of the *39K* and *gp64* promoters greatly reduced runoff transcription (lanes 4 and 8). Likewise, deletions that removed the UAR and TATA elements of both promoters greatly reduced runoff RNAs (Fig. 6B, lanes 5 and 9). Nonetheless, very low levels of runoff transcripts were consistently detected for each of the TATA-less *39K* and *gp64* promoters (Fig. 6B, lanes 4, 5, 8, and 9), as revealed by direct comparison to background levels from DNA templates containing only the respective UARs (lanes 6 and 10). Most notable was the higher yield of runoff transcripts from the TATA-less *ie-1* promoter (lane 2) compared with those of the TATA-less *39K* and *gp64* promoters. Thus, these data suggested that although the conserved CAGT motif is sufficient for low-level transcription from each promoter early during infection, the motif was most active within the context of the *ie-1* promoter.

Inhibition of late gene expression reduces accumulation of late *ie-1* transcripts. Unlike the transcripts from many Ac*M*NPV early genes, CAGT-initiated *ie-1* RNAs persist late in infection (30, 40). To assess the contribution of late transcription from the CAGT motif in maintaining *ie-1* RNA levels late in infection, cells infected with recombinant vIE1-FCAT were incubated in medium containing aphidicolin. This DNA synthesis inhibitor blocks Ac*M*NPV DNA replication and prevents late-virus gene expression (44). Levels of CAT-specific RNA were measured at intervals after infection by primer

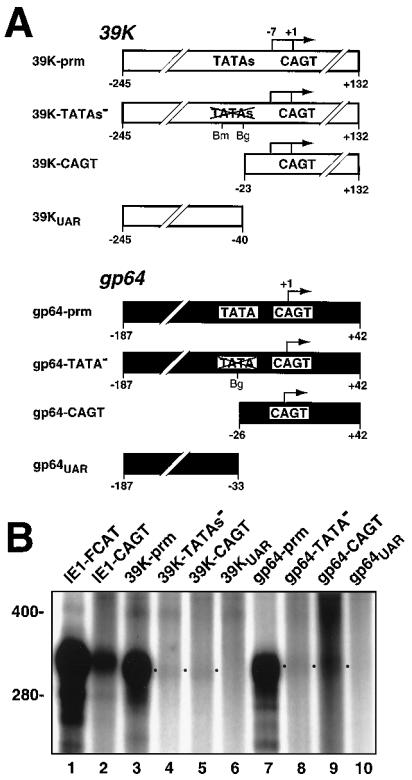

FIG. 6. In vitro transcription from TATA-less *39K* and *gp64* promoters. (A) Organization of Ac*M*NPV *39K* and *gp64* promoters. Plasmid 39K-prm contains the wild-type *39K* promoter from nucleotides −245 to +132 relative to the proximal RNA start (+1), consisting of a CAGT motif. In plasmid 39K-TATAs⁻, the proximal and distal TATA elements were eliminated by replacement with sequences containing *Bam*HI and *Bgl*II sites, respectively. In plasmid 39K-CAGT (nucleotides −23 to +132), both TATA elements were deleted. Plasmid gp64-prm contains the wild-type *gp64* promoter from nucleotides −187 to +42 relative to the CAGT start site (+1). The TATA element was eliminated by nucleotide replacement in plasmid gp64-TATA⁻ or deleted in plasmid gp64-CAGT (−26 to +42). Plasmids 39K$_{UAR}$ (−245 to −40) and gp64$_{UAR}$ (−187 to −33) contain only the *39K* and *gp64* UARs, respectively. Bg, *Bgl*II; Bm, *Bam*HI. (B) In vitro transcription. SF21 nuclear extracts derived from cells 4 h after infection were programmed with equivalent amounts (750 ng) of the indicated DNA templates. Proper transcriptional initiation yielded radiolabeled runoff RNAs of 333, 324/332, and 319 nucleotides from the *ie-1*, *39K*, and *gp64* promoters, respectively (denoted by dots).

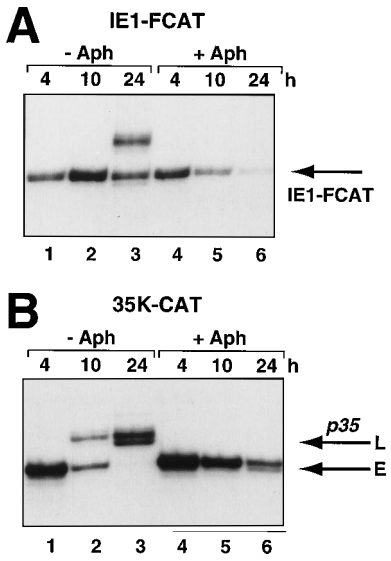

FIG. 7. Effect of aphidicolin on accumulation of vIE1-FCAT and v35K-CAT RNAs. SF21 cells were infected with recombinant virus vIE1-FCAT (A) or v35K-CAT (B) and were incubated with (+) or without (−) 5 μg of aphidicolin (Aph) per ml of growth medium. Total intracellular RNA was isolated at the indicated times (in hours) after infection and subjected to primer extension quantitation by using equivalent amounts (7 μg) of RNA and the 5′-end-labeled CAT primer as described in the legend to Fig. 2. Arrows denote the extension products specific to IE1-FCAT, early (E) *p35*-CAT, and late (L) *p35*-CAT RNAs.

extension assays. In the absence of aphidicolin, steady-state levels of IE1-FCAT RNA reached a maximum by 10 h and declined slightly by 24 h (Fig. 7A, lanes 1 to 3). In the presence of aphidicolin, IE1-FCAT RNAs peaked earlier (4 h) but declined sharply throughout infection (Fig. 7A, lanes 4 to 6). A longer 24-h RNA (lane 3) that maps 9 to 11 bp upstream from the CAGT motif also disappeared; the level of this late IE1-FCAT RNA was variable (Fig. 8 and 9) (40). In a confirmation of the capacity of aphidicolin to block late transcription, extension products specific to 24-h CAT RNAs from the late promoter (TTAAG) of *p35* were not detected in drug-treated cells infected with v35K-CAT (Fig. 7B); this Ac*M*NPV recombinant contains the *p35* promoter fused to the CAT gene inserted at the polyhedrin locus (13). Since the levels of 4-h CAT RNA from either the *ie-1* or the *p35* promoter were comparable with or without the drug (Fig. 7, lanes 1 and 4), aphidicolin had no effect on the early activity of either promoter. Thus, the reduction in steady-state levels of late *ie-1* RNA was due to inhibition of late-gene transcription, DNA replication, or both.

The CAGT motif is required for maximal steady-state levels of late *ie-1* RNAs. The reduction of late *ie-1* RNAs by aphidicolin, combined with the 1- to 3-h half-lives of early *ie-1* RNAs (22, 40), suggested that the *ie-1* promoter is active late in infection to maintain RNA levels. To examine the role of the CAGT motif in late *ie-1* transcription, we tested the effect of CAGT mutations (Fig. 2A) on steady-state levels of 24-h RNAs from Ac*M*NPV recombinants (Fig. 8). Compared with the wild-type motif (lane 1), replacement of nucleotides −3 and −1 [CAGT(−3,−1)] slightly reduced IE1-FCAT RNAs initiated from the CAGT motif (lane 2). All other mutations [CAGT(−1,+1), CAGT(+2,+3), and CAGT(−1/+3)] greatly reduced steady-state levels of late RNAs (Fig. 8, lanes 3 to 5).

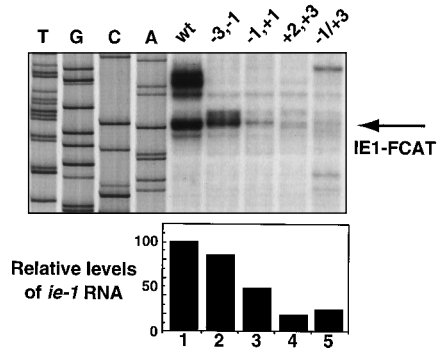

FIG. 8. Effect of CAGT mutations on levels of late *ie-1* transcripts. Total RNA was isolated 24 h after infection with recombinant viruses (multiplicity of infection of 20) containing the indicated nucleotide replacements within the CAGT motif of the IE1-FCAT promoter (see Fig. 2A). Equivalent amounts (14 μg) of RNA were quantitated by primer extension by using a 5′-end-labeled CAT primer as described in the legend to Fig. 2; the arrow denotes the extension products specific for IE1-FCAT RNA. Wt, wild type. The histogram (below) shows the levels of IE1-FCAT RNAs normalized to that of *p35* at the same time.

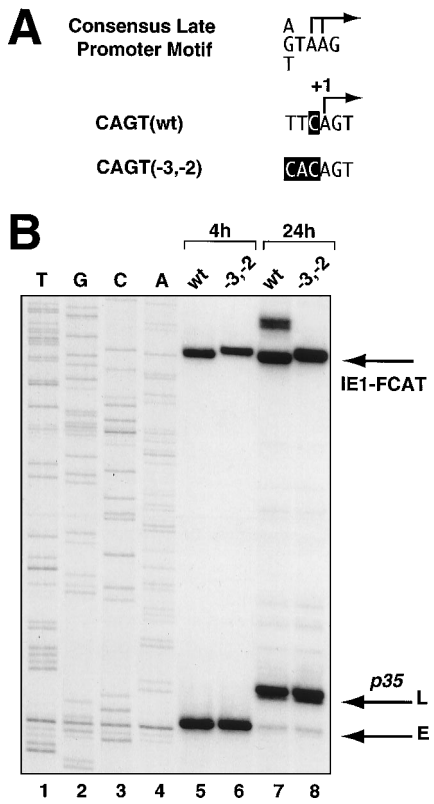

FIG. 9. Effect of nucleotides −3 and −2 on CAGT-mediated *ie-1* transcription. (A) Comparison of the baculovirus late promoter consensus with the *ie-1* CAGT motif. RNAs (arrows) initiate from the dinucleotide AA within the late consensus promoter (TAAG) and the dinucleotide CA within the *ie-1* CAGT motif (wt). In virus mutant vCAGT(−3,−2), the nucleotides TT (−3 and −2) were replaced with CA. Mismatches with the late consensus promoter are outlined in black. (B) Primer extension of early and late RNAs. Equivalent amounts (14 μg) of total RNA isolated 4 and 24 h after infection with viruses vIE1-FCAT and vCAGT(−3,−2) were quantitated by primer extension by using the 5′-end-labeled CAT primer as described in the legend to Fig. 2. Arrows denote the extension products specific to IE1-FCAT, early (E) *p35*, and late (L) *p35* RNAs.

Because of higher background levels of RNAs at 24 h, the quantitation shown (Fig. 8, histogram) is an underrepresentation of the actual reduction of CAGT-initiated RNAs. All mutations eliminated the 24-h RNA mapping further upstream.

Except for a single nucleotide mismatch, the *ie-1* sequence at the CAGT motif is identical to the consensus motif (TAAG) for baculovirus late promoters (Fig. 9A). Thus, to test whether the *ie-1* CAGT motif functions as a weak consensus promoter late in infection, recombinant virus vCAGT(−3,−2) in which substitutions of nucleotides −3 and −2 reduced the similarity to the late consensus motif was constructed (Fig. 9A). However, both early (4 h) and late (24 h) after infection, the steady-state levels of IE1-FCAT RNA from vCAGT(−3,−2) were comparable to that from vIE1-FCAT containing the wild-type CAGT motif (Fig. 9B). The mutation caused a 2-bp shift in the RNA start site and eliminated the longer 24-h RNA (Fig. 9B, lanes 7 and 8). Thus, although the CAGT motif is necessary for the maximum levels of late *ie-1* RNAs, this analysis suggested that it does not function as a weak consensus motif that is active in late baculovirus promoters.

DISCUSSION

The *ie-1* CAGT motif functions as a transcription initiator element. An initiator is a DNA sequence that overlaps the RNA start site of an RNA polymerase II promoter and determines the position of transcriptional initiation. In the absence of a TATA element, the initiator is sufficient to determine the position of the RNA start site (47). In the case of TATA-containing promoters, the initiator is a key component of the basal promoter since it can cooperate with the TATA element to stimulate transcription (42, 47). On the basis of mutational analyses, the consensus sequence $PyPyA_{+1}N(T/A)PyPy$ has been proposed for vertebrate initiators (23), while the related consensus sequence (A/C/T)CA(G/T)T was compiled by surveying invertebrate initiators (10). The high degree of conservation among initiators and the existence of a functional consensus sequence suggest that the initiator is recognized by a universal transcription factor(s) (23). Indeed, current evidence suggests that initiators interact with a component of the TFIID transcription complex (24, 41, 48, 52).

By constructing Ac*M*NPV recombinants with site-specific mutations in the *ie-1* promoter, we have demonstrated for the first time that a CAGT motif located at the RNA start site of a baculovirus gene functions as an initiator element. Our data indicated that the CAGT motif is a core promoter element which regulates the rate of *ie-1* transcription and defines the RNA start site. First, nucleotide replacements within the CAGT motif reduced the steady-state levels of early *ie-1* RNAs (Fig. 2B) and the rate of *ie-1* transcription, as measured by the yield of runoff transcripts in SF21 nuclear extracts (Fig. 2C). Maximum *ie-1* promoter activity was observed when the CAGT motif most closely resembled the consensus initiator. Simultaneous replacement of the four nucleotides constituting the core of the initiator motif reduced in vivo and in vitro transcription rates from the full-length *ie-1* promoter by 10-fold or more (Fig. 2). Since the *ie-1* TATA element was present, it was not surprising that mutagenesis of the core motif failed to abolish transcription. As shown previously (23), initiator mutations have the greatest impact in the absence of a fully functional TATA element, suggesting that concomitant protein interactions at the TATA element may compensate for less-than-optimal interactions at the initiator element. Deletion of upstream and downstream *ie-1* promoter sequences demonstrated that nucleotides −6 to +11, including the core CAGT motif, were sufficient for proper transcriptional initiation (Fig. 5). In the case of a TATA-containing promoter, simultaneous deletion of the CAGT motif and DAR eliminated *ie-1* transcription (40). Thus, the TATA element was not sufficient or required to direct proper *ie-1* transcription. Nonetheless, the ~50-fold stimulation of *ie-1* transcription by the TATA element (Fig. 3) suggested that it cooperates with the CAGT initiator to regulate *ie-1* promoter activity. Likewise, sequences within the 5′ noncoding leader region of *ie-1*, including the DAR (+11 to +24), contributed to CAGT-initiated transcription (Fig. 5). Downstream elements also stimulate the activity of other known initiators (5, 14, 36, 42).

Further support for initiator function of the *ie-1* CAGT motif was the finding that TATA-less promoter activity was augmented by a heterologous upstream activating element, a property common to vertebrate initiators (5, 8, 36, 47, 48). When placed upstream of the TATA-less *ie-1* promoter, the *p35* UAR stimulated CAGT-initiated transcription by as much as 3.5-fold (Fig. 3 and 4). As the distance between the CAGT motif and UAR was altered, the level of stimulation, but not the RNA start site, varied. Thus, the UAR failed to affect the site of initiation, further supporting the role of the CAGT initiator in defining the RNA start. Despite its capacity to stimulate transcription from TATA-containing promoters (40, 45), the Ac*M*NPV *hr*5 enhancer had no effect on CAGT initiator activity (Fig. 3). *hr*5 also failed to stimulate transcription

from the U3-R promoter of the long terminal repeat of the lepidopteran retrotransposon TED (45); this promoter lacks a TATA element but contains an initiator-like sequence (G<u>CA$_{+1}$TTCC</u>) at the RNA start site (15). Thus, early transcriptional enhancement by *hr5* may require the presence of a functional TATA element.

Functional significance of the CAGT initiator for *ie-1* transcription. The *ie-1* promoter is active immediately upon infection (19, 30, 40). This rapid expression supports the view that IE1 is an essential transregulator that is required early in infection for proper activation of IE1-dependent viral promoters and initiation of DNA replication (19, 29, 31, 37, 40, 43). Our data indicated that most, if not all, of the activity of the *ie-1* promoter early in infection is provided by a cooperation between the CAGT initiator, TATA element, and DAR; the *ie-1* UAR has little influence (40). The CAGT motif plays a key role by contributing to RNA start site selection and stimulating early *ie-1* promoter activity. Because of its sequence identity with other characterized initiators, it is possible that the *ie-1* CAGT initiator is recognized by a universal and possibly ubiquitous transcription factor from the host, much as the TATA element is recognized by the universal TATA-binding protein. Indeed, host factors were sufficient for TATA-less initiator activity from the CAGT motif in transcription reaction mixtures consisting of nuclear extracts from uninfected cells (Fig. 3C). Combined with the TATA element, the CAGT initiator may function to ensure immediate *ie-1* transcription by recruiting pre-existing, universal host factors. A viral strategy in which *cis*-acting stimulatory elements are used to recruit ubiquitous host factors would also expedite *ie-1* expression and virus replication in a wider variety of host cell types. The CAGT motif may therefore confer expanded tissue tropism and host range. Support for such a hypothesis awaits identification of recognition factors for lepidopteran initiators.

Does the CAGT motif function as an initiator for other baculovirus genes? The demonstration of *ie-1* CAGT initiator activity suggested that identical motifs at the RNA start sites of other baculovirus genes function similarly. Indeed, previous mutational studies have shown that the CAGT motif stimulates expression from the baculovirus *ie-2*, *39K*, and *gp64* promoters (2, 7, 17). In the case of a 43-bp synthetic promoter from *gp64* or *Orgyia pseudotsugata* nuclear polyhedrosis virus (2), replacement of the CAGT motif reduced the rate of transcription in nuclear extracts from uninfected cells. However, TATA-independent initiation from the CAGT motif was not detected (2). Using the *gp64* and *39K* promoters of Ac*M*NPV as templates, we detected low-level TATA-independent transcription in nuclear extracts from virus-infected SF21 cells (Fig. 6). Replacement or deletion of the TATA elements of both promoters greatly reduced, but did not eliminate, in vitro transcription from the CAGT motif. Our findings were consistent with *gp64* and *39K* CAGT initiator function and confirmed the stimulatory role of their TATA elements (2, 17). In support of the CAGT initiator activity from *gp64* of Ac*M*NPV, a recent study has shown that in vitro runoff transcripts initiate from the CAGT motif of a full-length, TATA-less *gp64* promoter of Op*M*NPV (28). Most striking in our study was the significantly higher in vitro transcription activity of the CAGT motif from the TATA-less promoter of *ie-1* compared with those of *gp64* and *39K* (Fig. 6). The strength of the *ie-1* CAGT initiator may be due, in part, to the nearly perfect match of the core and flanking sequences with the preferred initiator binding sequence for *Drosophila* TFIID (G/A/<u>T</u>)(<u>C</u>/T)<u>A</u>(T/<u>G</u>)<u>T</u>G and the consensus initiator <u>PyPyA$_{+1}$N(T</u>/A)<u>Py</u>Py (matches underlined) (23, 41). Moreover, *ie-1* initiator activity was strengthened by the *ie-1* DAR (Fig. 5). Thus, although numerous baculovirus genes possess a CAGT motif at their RNA start sites, the overall strength of the motif as an initiator may depend on the presence and influence of nearby regulatory motifs. It is noteworthy that many baculovirus genes lack an obvious CAGT motif at their RNA start sites. For these genes, the TATA element may suffice to position the RNA start site, as is the case for the *p35* gene of Ac*M*NPV (13).

Role of the CAGT motif in late *ie-1* transcription. *ie-1* is unique among characterized Ac*M*NPV genes since its CAGT-initiated transcripts are present both early and late in infection (30, 40). Transcription of early genes usually declines late in infection, whereas late RNAs are initiated from the consensus late promoter element consisting of a TAAG motif (for reviews, see references 4 and 38). On the basis of the relatively short half-lives (1 to 3 h) of early *ie-1* RNAs (22, 40), the presence of *ie-1* RNAs late in infection suggested that the *ie-1* CAGT motif also functions as a late promoter element. In this study, nucleotide replacements within the CAGT motif reduced steady-state levels of late IE1-FCAT RNAs of Ac*M*NPV recombinants (Fig. 8). Thus, the CAGT motif directly or indirectly affected the level of late *ie-1* RNAs. Additional mutations that reduced sequence similarity with the consensus late (TAAG) promoter, while preserving the CAGT core, had no effect on the steady-state levels of early or late *ie-1* RNAs initiated from the CAGT motif (Fig. 9B). Thus, the CAGT motif does not function as a weak consensus late (TAAG) promoter. Lastly, the DNA synthesis inhibitor aphidicolin prevented accumulation of late *ie-1* transcripts, without affecting early promoter activity (Fig. 7). Thus, late transcription or viral DNA replication was required to maintain normal levels of late *ie-1* RNA.

Collectively, our data are consistent with the *ie-1* CAGT motif functioning as a novel late promoter that lacks a consensus TAAG motif. It is noteworthy that an initiator element also directs late transcription from polyomavirus (55). In support of the existence of distinct regulatory elements that regulate late CAGT initiator activity, we previously observed that maintenance of late *ie-1* RNA levels requires 5′ noncoding sequences (+11 to +36) that extend further downstream from those required for early promoter activity (40). Although we cannot yet rule out the possibility that these downstream sequences stabilize late *ie-1* RNAs, they had no effect on early RNA stability (40). Our data do not exclude an alternative mechanism whereby late *ie-1* RNA levels are maintained by a late viral factor (e.g., RNA-binding protein) that selectively stabilizes *ie-1* RNAs synthesized early in infection. Expression of such a novel factor would be inhibited by aphidicolin and could account for the loss of late *ie-1* RNA in the presence of the drug (Fig. 7). Nonetheless, the stability of early *ie-1* RNAs would have to be mediated by a factor interacting with nucleotides +1 to +74, since both native *ie-1* and IE1-FCAT RNAs persist late in infection (40). Lastly, the *ie-1* initiator may be recognized by host RNA polymerase II late in infection, despite the shutoff of many early Ac*M*NPV promoters at this time. Future experiments should distinguish these interesting possibilities.

ACKNOWLEDGMENTS

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