Baculovirus Gene ME53, Which Contains a Putative Zinc Finger Motif, Is One of the Major Early-Transcribed Genes

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We have been investigating the first viral genes to be transcribed in *Spodoptera frugiperda* cells after the infection with *Autographa californica* nuclear polyhedrosis virus in order to identify regulatory proteins required for the activation of early and/or late gene transcription. By screening a cDNA library from polyadenylated RNA transcribed at 1 h postinfection, we identified a gene which is designated ME53. This newly identified gene is located upstream of the late p74 gene and forms a divergent promoter unit with the immediate-early gene IE0. The determination of the nucleotide sequence of ME53 has revealed an open reading frame encoding a gene product of 53 kDa. Furthermore, the sequence data suggest a putative DNA binding motif, a zinc finger, whose functional significance has yet to be shown. Transcription of ME53 does not require previous viral protein synthesis, and during infection, the level of promoter activity seems to be independent of *trans*-acting viral factors, as suggested by transient expression studies.

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has a double-stranded DNA genome of about 130 kb. During the complex replication cycle of AcNPV, which entails the production of two virus phenotypes, viral gene expression is regulated in a cascade fashion (for reviews, see references 2, 8, and 11). Prior to viral DNA synthesis, the early set of genes is transcribed by the host RNA polymerase II (12, 19). However, transcription of the majority of late and very late genes is mediated by an α -amanitin-resistant RNA polymerase (12, 47). The activation of late and very late gene transcription is thought to be dependent on early viral gene products and on DNA replication. This statement is based on several findings, such as the characterization of mutants which are defective in late gene expression (14, 31, 35); the sensitivity of late gene transcription to cycloheximide and aphidicolin, inhibitors of protein synthesis or DNA replication (38); or the transcriptional activation of late and very late promoters by viral factors (17, 20). While much progress has been made in the past several years in identifying *cis*-acting elements in early, late, and very late promoters, a detailed understanding of *trans*-acting viral factors regulating the cascade of gene expression still remains to be elucidated. The gene product of the immediate-early gene 1 (IE1) has been shown to stimulate other baculovirus gene promoters (3, 15). Recently, *trans*-acting properties in transient expression assays have also been demonstrated for the IE0 gene product, the spliced form of the IE1 gene, and for the product of the immediate-early gene N (IEN) (4, 22).

In the course of research on early regulatory genes, we identified the major early-transcribed PE38 gene (24). In the present study, we have analyzed another major early transcript whose corresponding gene is designated ME53. The 3' terminus of the ME53 gene is located upstream of the late p74 gene (25), and the ME53 promoter forms a divergent transcription unit with the IE0 promoter (6). We have previously reported that the PE38 gene (24) is transcribed in the opposite direction to the IEN gene (4). Comparing the transcriptional patterns of the PE38/IEN and ME53/IE0/IE1

genes in the course of infection, we conclude that all five genes are transcriptionally active as early as 1 h postinfection (p.i.). Activity decreases at 26 h p.i. As shown by transient expression studies in uninfected *Spodoptera frugiperda* cells, the ME53 promoter is transcribed in the absence of any other viral protein. These results are similar for the promoters of IE1, IE0, IEN, and PE38 as recently described (4, 15, 22, 24).

MATERIALS AND METHODS

Cells and virus. *S. frugiperda* IPLB21 cells (45) and *Trichoplusia ni* TN-368 cells (18) were grown as monolayer cultures in TC100 medium supplemented with 10% fetal calf serum (13). The AcNPV plaque isolate E (44) was used for studies on the time course of infection as described earlier (24). Time zero was defined as the time when the AcNPV inoculum was added to the cells.

Plasmid construction. The *Hind*III G fragment, 90.3 to 96.5 map units (m.u.), or the *Hind*III P fragment, 88.7 to 90.3 m.u., of AcNPV isolate E DNA were cloned into the plasmids pBluescript KS(+) or pBR322. A subclone of the *Hind*III P fragment, the *Xho*I-*Hind*III fragment, 90.1 to 90.3 m.u., was inserted into the vector pBluescript KS(+). A subclone of the *Hind*III G fragment, a *Hpa*II-*Hpa*II fragment, which included the promoters of ME53 and IE0, was blunt ended by fill-in reaction with Klenow polymerase and cloned into the blunt-ended *Cla*I site of the pBluescript KS(+) vector. This construct was designated pHindG-HH.

Two ME53 promoter fragments which were generated by polymerase chain reaction (39) and by using cloned DNA templates were fused to the prokaryotic chloramphenicol acetyltransferase gene (CAT) in the construct pBLCAT3 (27). Each of the promoter fragments, comprising 202 or 74 nucleotides of the ME53 promoter, was inserted into the *Bgl*II and *Pst*I sites of the pBLCAT3 plasmid, thus generating the constructs pME53-CAT202 and pME53-CAT74, respectively. The pIE0-CAT plasmid was obtained by inserting the *Hpa*II-*Hpa*II fragment in front of the CAT gene. In this construct, CAT activity was driven by the IE0 promoter.

Sequencing and computer analyses. The nucleotide se-

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quences of the *XhoI-HindIII* fragment *HindIII* P, of part of the *HindIII* G fragment, and of the cDNA clone ME53, designated clone 104, were determined by the chain termination method (40) by using the T7 sequencing kit (Pharmacia). Furthermore, the nucleotide sequences of the 5' and 3' ends of six additional ME53 cDNA clones, designated clones 5, 95, 99, 103, 106, and 107, were determined. For the sequencing of either strand of the double-stranded templates, 21 synthetic oligodeoxyribonucleotides of 17 nucleotides (nt) in length, T7 17-mer, and T3 17-mer (Stratagene) were used as primers.

The DNA sequences and the deduced amino acid sequences of the ME53 open reading frame (ORF) were compared with sequences in the most recent GenBank/EMBL (release 31, June 1992) and SWISSPROT data bases by using the FASTA program (36). The analyses were performed by EMBL (Heidelberg, Germany).

cDNA synthesis. The synthesis of cDNAs from poly(A)⁺ RNA isolated from AcNPV-infected *S. frugiperda* cells 1 h p.i. was described earlier (24).

Transcriptional mapping. AcNPV-infected cells were harvested at various times after inoculation. The preparation of cytoplasmic RNA, of poly(A)⁺ RNA, and the Northern (RNA) blot analyses were performed as recently published (24).

The 5' end of the early ME53 transcript was mapped by the RNase protection technique (29, 48). Specific RNA probes were synthesized in vitro from the pHindG-HH plasmid, which was linearized with *HindIII*. In vitro transcription reactions by T3 RNA polymerase were performed in the presence of [³²P]UTP (32), and the RNA transcripts were purified by electrophoresis on 5% polyacrylamide gels. For RNase protection, 10 µg of cytoplasmic RNA or 100 ng of poly(A)⁺ RNA was annealed to the specific RNA probe (~1 × 10⁵ Cerenkov cpm) at 46°C overnight. The hybrids were treated with 7 µg of RNase A and 35 U of RNase T₁ in 10 mM Tris-HCl, 5 mM EDTA, and 300 mM NaCl for 45 min at 37°C. After proteinase K treatment and phenol-chloroform extraction, the hybrids were analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea.

Transient-expression assays. Transfection experiments were performed in uninfected *S. frugiperda* or TN-368 cells at 15 to 20 h after seeding the cells, and CAT activity was assayed as detailed earlier (23). The cell cultures were infected with AcNPV at a multiplicity of approximately 10 PFU per cell and transfected at 4 h p.i.

Nucleotide sequence accession number. The nucleotide sequence accession number of the ME53 gene is L05914.

RESULTS AND DISCUSSION

Transcriptional analysis of the ME53 gene. We investigated the early transcripts in the region of the *HindIII* G fragment (90.3 to 95.5 m.u.) by screening a cDNA library prepared from poly(A)⁺ RNA isolated 1 h p.i., as published recently (24). We found cDNA clones including the ORF of IE1 as well as of the spliced form IE0. Our sequence data confirmed the IE1 and IE0 sequences determined by other groups (6, 16), except for one base pair exchange at the splice site of IE0. This exchange did not alter the amino acid sequence (data not shown). We newly identified a set of cDNA clones which mapped to the left end of the *HindIII* G fragment and corresponded to the early 1.6-kb transcript, as shown by hybridization experiments (Fig. 1b). The early-transcribed gene was designated ME53; its temporal expression pattern is shown in Fig. 1b. We prepared poly(A)⁺ RNA from

FIG. 1. Northern blot analysis of ME53- and IE1/IE0-specific transcripts. Poly(A)⁺ RNA was isolated from AcNPV-infected *S. frugiperda* cells at 0.5 h (lane 0.5), 1 h (lane 1), 2 h (lane 2), 6 h (lane 6), 12 h (lane 12), and 26 h (lane 26) p.i. Samples of 9 µg (lanes 0.5 and 1), 4.5 µg (lane 2), and 2.5 µg (lanes 6, 12, and 26) of poly(A)⁺ RNA were loaded and electrophoresed on a 1.2% agarose gel. After transfer to a nitrocellulose filter, the RNA was hybridized to a IE1-specific cDNA clone (a) and rehybridized to the ME53-specific cDNA clone 104 (b) and to the *Drosophila* actin probe pAct-5c (c). Positions of the DNA size markers (lane M) are indicated on the right. The sizes of the IE1/IE0- and ME53-specific transcripts are marked by arrowheads.

AcNPV-infected cells at different times p.i. and analyzed the RNA on Northern blots by hybridizing to a ME53-specific cDNA (cDNA clone 104). A transcript of approximately 1,600 nt in length was already apparent at 0.5 h p.i., more abundant at 2 h p.i., and still present at 26 h p.i. Transcripts which overlapped the ME53 gene were detectable at 6 and 12 h p.i. The approximate sizes of these more weakly expressed RNAs were 1,800, 2,200 and 3,500 nt. Overlapping transcripts appearing during the course of AcNPV infection were described for many regions of the AcNPV genome (10, 26, 28, 33, 37) and are thought to be related to gene regulatory mechanisms (34; for a review, see reference 11).

The transcriptional pattern of the IE1/IE0 genes was similar to that of the ME53 gene, although the IE1 gene transcript of 1.9 kb and the IE0 gene transcript of 2.1 kb were less abundant at 0.5 and 1 h p.i. than the ME53 gene transcript (Fig. 1a). By hybridizing to IE1- or IE0-specific cDNA clones, we detected no decrease of the IE0-specific transcript at 6 and 12 h p.i. (Fig. 1a) in contrast to previously described Northern blot analyses (6). Additional transcripts which overlapped the IE1 gene were detectable at 12 h p.i. and corresponded to those reported earlier (6, 16).

Nucleotide sequence of the ME53 gene and mapping of its early 5' terminus. By using a series of synthetic primers, the nucleotide sequences of both strands of the ME53-specific cDNA clone 104 were determined. The genomic nucleotide sequence of the ME53 gene was assessed from the *HindIII* G and the *XhoI-HindIII* fragments. Figure 2 presents the nucleotide sequence of the leftward-transcribed ME53 gene according to the conventional orientation of the AcNPV genome (46). This region includes one major ORF potentially encoding a 449-amino-acid polypeptide with a predicted molecular mass of 53 kDa. The amino acid sequence of the

FIG. 2. Nucleotide sequence of the ME53 region. The DNA sequence of 1,800 nt and the predicted amino acid sequence are shown in genomic orientation. The 5' and 3' ends of seven ME53-specific cDNA clones are indicated by the numbers of the cDNA clones 107, 104, 106, 95, 99, 5, and 103. The 5' ends of the early ME53 transcript determined by RNase protection, the early IE0 transcript (6), and the major transcriptional start site of the p74 gene (25) are depicted by arrows. Potential polyadenylation signals are overlined; TATA consensus sequences and a GATA motif upstream of the ME53 RNA initiation site are boxed. The conserved TATA elements 5' of the IE0 and the ME53 genes are marked by broken lines. The cysteine residues of a putative zinc finger motif in the ME53 ORF are indicated by closed boxes. The location of the ME53 ORF in close vicinity to the ORFs of the p74 and IE0 genes is shown below.

ME53 ORF showed no strong sequence homology with any other known protein in the SWISSPROT data base. Further analyses of the amino acid sequence data suggest a zinc finger motif (CX₂CX₁₃CX₂C) at the carboxy terminus of

ME53 (Fig. 2). Zinc finger motifs consisting only of clustered cysteine residues have been observed in the nuclear receptor family (for a review, see reference 41) and are thought to be essential for sequence-specific recognition of DNA (9).

FIG. 3. RNase protection analysis of early ME53 gene transcription. Cytoplasmic RNA (10 μ g, lane 2) or poly(A)⁺ RNA (100 ng, lane 3) isolated 2 h p.i. were hybridized to the RNA probe of 351 nt (see Material and Methods). The hybrids were analyzed on a 6% polyacrylamide sequencing gel. The protected hybrid of 87 nt and the size of the RNA probe (lane 1) are indicated by arrowheads. Positions of the DNA size markers (lane M) are shown at the right.

Whether the putative ME53 zinc finger motif plays any functional role has yet to be shown. At the amino terminus of the ME53 ORF there is a stretch of 40 amino acids containing approximately 26% proline residues. Proline-rich regions were observed in a variety of viral and cellular genes; the proline-rich domain of the human CTF/NF-1 gene family has been proposed to represent a novel class of transcriptional activator motifs (30).

The 5' and 3' nucleotide sequences of the six additional ME53-specific cDNA clones, 5, 95, 99, 103, 106, and 107, were determined (Fig. 2). Interestingly, the 3' ends of the two cDNA clones 106 and 107 differed in 23 and 47 nt, respectively, from the 3' ends of the majority of the cDNA clones (clones 5, 95, 99, 103, and 104). Since four polyadenylation sites were following the stop codon of the ME53 ORF (Fig. 2), our sequence data suggest the usage of different polyadenylation sites early after infection. Alternative polyadenylation in the 3' untranslated region of the amyloid protein precursor has been demonstrated to modulate its translational efficiency (7).

It is worth mentioning that 3 of the AATAAA sequences

were located downstream or near the transcriptional start site of the p74 gene (Fig. 2), which was maximally transcribed at 16 to 20 h p.i. (25). Whether usage of different polyadenylation sites at the 3' end of the ME53 ORF relates to the transcriptional regulation of the p74 gene has still to be shown.

The ME53 and IE0 genes are divergently transcribed, and their early transcriptional start sites are separated by approximately 163 bp. Late-spliced transcripts of the IE1 gene have been shown to initiate upstream as well as downstream of the early IE0 RNA initiation site (21).

The 5' end of the early ME53 transcript was mapped by RNase protection analyses (Fig. 3). Cytoplasmic or poly(A)⁺ RNA isolated 2 h p.i. was hybridized to a specific RNA probe of 351 nt in length which was synthesized in vitro from the linearized pHindG-HH plasmid. The occurrence of a protected fragment of approximately 87 nt indicates an early transcriptional start site at a point 36 to 41 nt upstream of the predicted ATG start codon. This start site is homologous to the IE0 initiation site and includes a CAGT motif (CAGTATC) which is a conserved sequence element close to the transcriptional start site of several early baculovirus genes (1, 5, 6, 15, 24, 42, 43). Furthermore, a consensus TATA box element 25 to 30 bp upstream from the 5' terminus of the ME53 or IE0 gene is conserved over a stretch of 11 bp (TATATAAGCC).

The ME53 promoter includes the binding motif GATA, which has been shown to compete for an insect GATA-like protein in nuclear extracts of *S. frugiperda* cells (SfNP-1) (23). The functional significance of the GATA motif in the ME53 promoter has yet to be determined.

ME53 promoter activity in *S. frugiperda* and in TN-368. To investigate whether the ME53 gene requires previous viral protein synthesis for its transcriptional activity, we performed transient-expression assays. The ME53/IE0 promoter unit was placed upstream of the CAT gene and transfected into uninfected or AcNPV-infected *S. frugiperda* cells. The two ME53 promoter constructs which differ in length are outlined in Fig. 4b; the pME53-CAT202 plasmid comprises the entire promoter region of ME53/IE0, and the pME53-CAT74 construct lacks the sequences upstream of the TATA consensus sequence.

In uninfected *S. frugiperda* cells, the construct pME53-CAT202 was active (Fig. 4a), and no significant increase in CAT expression was apparent when *S. frugiperda* cells were infected with AcNPV prior to transfection (Fig. 4c). The shorter pME53-CAT74 construct was nearly silent in uninfected as well as infected *S. frugiperda* cells (Fig. 4c). These results suggest that ME53 promoter sequences upstream of the TATA box are needed for transcriptional activity which is mediated by *S. frugiperda* host cell factors.

When transfection experiments were performed in the permissive cell line TN-368, the shorter pME53-CAT74 construct was as efficiently expressed as pME53-CAT202 (Fig. 4d). These data imply that the truncated form of the ME53 promoter was sufficient to be recognized by the transcriptional machinery of the permissive cell line TN-368.

As a control, we also tested promoter activities of the IE0 and PE38 genes in uninfected insect cells. From previous work it is known that the IE0 and PE38 gene promoters are active in the absence of any other viral gene product (22, 24). Transient-expression assays in *S. frugiperda* (Fig. 4a) or TN-368 cells (Fig. 4d) indicated that the levels of CAT expression were comparable among the constructs pME53-CAT202, IE0-CAT, and PE38-CAT207.

Since we did not investigate promoter activities in the

FIG. 4. CAT activity of ME53 promoter constructs in *S. frugiperda* and TN-368 cells. *S. frugiperda* (a and c) or TN-368 cells (d) were transfected with 15 μ g of each of the promoter constructs indicated and were harvested 43 h after transfection. Cell extracts were tested for CAT activity and [14 C]chloramphenicol and its acetylation products were separated by chromatography. The results of a commercial CAT enzyme control are shown. The construct pPE38-CAT207 was described earlier (23). (a) Transfections were performed in uninfected *S. frugiperda* cells. (b) Lengths of promoter fragments are indicated by heavy lines; the arrows indicate the ME53 or IE0 RNA initiation sites, to which numbering refers. (c) Transfection of uninfected (u) and AcNPV-infected (4 h p.i.) (i) *S. frugiperda* cells. (d) Transfection of uninfected TN-368 cells.

context of the viral genome, it cannot be excluded that the expression of the promoter constructs used only reflects basal promoter activities without additional upstream or downstream sequences which could be involved in the modulation of the transcriptional activity.

In summary, we have identified the ME53 gene which corresponds to the major early transcript of 1,600 nt after AcNPV infection. On the basis of the suggested DNA binding property, we postulate that ME53 might play a regulatory role in the course of infection. The ME53 promoter does not need any viral protein for its transcriptional activity in *S. frugiperda* or TN-368 cells, as demonstrated by transient-expression assays. However, first investigations on the sequence requirements for promoter activity suggest a different regulation in the permissive cell lines *S. frugiperda* and TN-368.

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