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Sequence and Temporal Appearance of the Early Transcribed Baculovirus Gene HE65

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We have identified the early transcribed HE65 gene by screening a cDNA library from polyadenylated RNA which was isolated at 1 h after infection of *Spodoptera frugiperda* cells with *Autographa californica* nuclear polyhedrosis virus (AcNPV). Nucleotide sequencing analysis of the HE65-specific cDNA clone reveals one open reading frame of 1,662 nucleotides from which a protein of 65 kDa in size can be predicted. The HE65 gene is located downstream of the late transcribed p80 gene and upstream of the homologous region *hr*4left, which overlaps the 5′ sequences of the HE65 gene. An HE65-specific transcript of about 1,800 nucleotides is detectable 2 h postinfection and remains stable during the late phases of infection. RNase protection and primer extension analyses demonstrate that transcripts from the early start site of HE65 continue to accumulate from 2 to 48 h postinfection, even in the presence of aphidicolin. Furthermore, transcriptional analysis of the HE65 gene indicates a lower intensity of early transcription in comparison with the very early transcribed genes IEN, PE38, and ME53.

The DNA genome of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has the capacity to encode about 100 genes. During productive infection in insect cell culture, these genes can be divided in different temporal classes that are coordinately and sequentially regulated (for reviews, see references 1 and 23). An additional RNA polymerase activity, characterized by its α-amanitin resistance and its induction in the course of infection, probably plays an essential role in the early-to-late phase transition (9, 32). It is unknown how viral gene products direct gene activation of different temporal classes in concert with host factors.

Our recent studies have focused on the identification and characterization of the most intensely transcribed early genes, assuming their possible involvement in regulatory events (16, 19). Here, we report on the newly identified gene HE65, whose transcriptional activity at 1 and 2 h postinfection (p.i.) is weaker than that of the major early transcribed genes PE38 and ME53.

The cDNA clones of all three genes, HE65, ME53, and PE38, have been isolated from the same cDNA library, which has been established from polyadenylated RNA prepared 1 h after infection of *Spodoptera frugiperda* cells with AcNPV, as recently described (19). The genes ME53 (16), IE1/IE0 (5, 13), IEN (2), and PE38 (19) are clustered in a region of 12 kb, and each of them forms an early divergent promoter unit (Fig. 1). In contrast, the location of the HE65 gene is thus far unrelated to other early transcriptional events (Fig. 1). Its 5′ terminus is located in close vicinity to the homologous region *hr*4left (10). The five homologous regions of the AcNPV genome include multiple *Eco*RI sites forming imperfect palindromic sequences (6). All five regions have been shown in transient expression assays to act as enhancers for some of the early viral promoters (3, 10, 14, 22). It has been suggested that the homologous regions serve as viral origins of replication (6, 17, 24).

The HE65 gene is in tail-to-tail orientation with the late transcribed p80 gene (21). The termination and the polyadenylation signals of the HE65 and p80 genes are separated by only 22 nucleotides.

MATERIALS AND METHODS

Cells, virus, and aphidicolin treatment. The conditions for cell culture of *S. frugiperda* IPLB21(SF21) (30) and for their infection with the AcNPV plaque isolate E (29) were described earlier (19). Time zero was defined as the time when the AcNPV inoculum was added to the cells. To inhibit AcNPV DNA replication, *S. frugiperda* cells were treated with aphidicolin (5 mg/ml; Sigma) at 1 h after AcNPV inoculation as described by Rice and Miller (26).

Plasmid construction and cDNA synthesis. The *Eco*RI Q fragment, 67.8 to 69.3 map units, of AcNPV plaque isolate E DNA was cloned into plasmid pBluescriptKS(+) (Stratagene). A subclone of the *Eco*RI Q fragment, the *Sty*I-*Eco*RI fragment comprising the 5′ sequence of the HE65 gene, was blunt ended by fill-in reaction with Klenow polymerase and inserted via a transfer vector into plasmid pBluescriptKS(+).

Synthesis of the cDNA library was described previously (19).

Sequencing and computer analyses. The nucleotide sequences of the HE65-specific cDNA clone 53 and the *Eco*RI Q fragment were determined by the chain termination method (27), using 17 synthetic oligodeoxyribonucleotides of 17 nucleotides (nt) in length as well as T7 and T3 primers (Stratagene) (19). The 5′ and 3′ ends of the two additional HE65-specific cDNA clones 18 and 64 were also sequenced.

The deduced amino acid sequence of the HE65 open reading frame (ORF) was compared with sequences in the most recent SWISSPROT data base (release 24) by using the FASTA program (25).

RNase protection and primer extension analyses. AcNPV-infected *S. frugiperda* cells were harvested to prepare cytoplasmic or poly(A)$^+$ RNA, which was analyzed by Northern (RNA) blotting. The 5′ end of the HE65-specific transcript was mapped by primer extension analyses and RNase pro-

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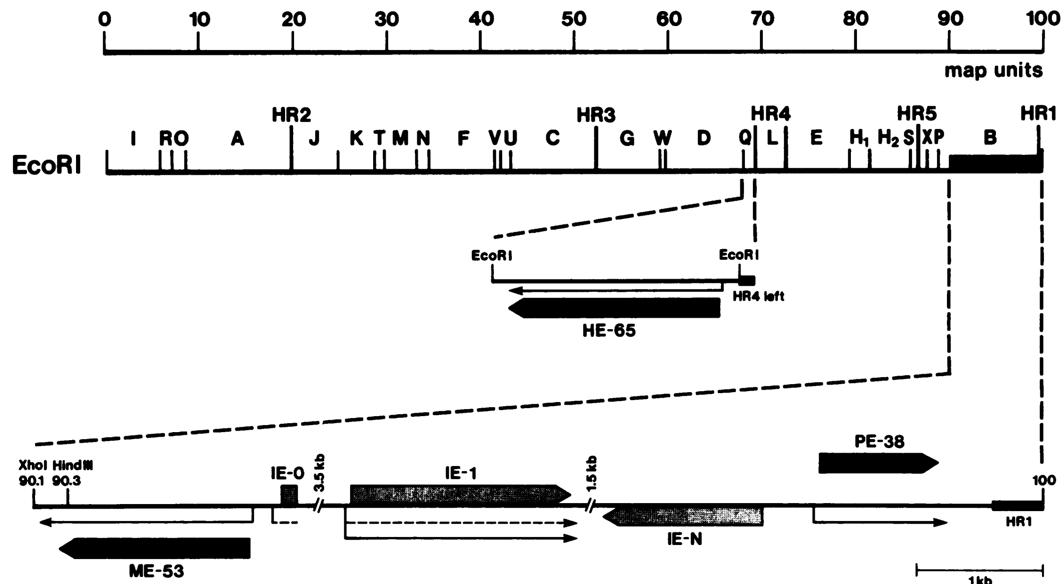

FIG. 1. Locations of the early genes HE65, ME53, IE1/IE0, IEN, and PE38 on the AcNPV genome. The linearized *Eco*RI map of the AcNPV plaque isolate E genome is shown. The five homologous regions (HR1 to HR5) are indicated, and the black bar represents a very early transcribed region of about 12 kbp. The directions of the HE65, ME53, IE1/IE0, IEN, and PE38 ORFs are shown by black or grey arrows. The early transcripts are designated by thin arrows, and the spliced IE0 transcript is marked by a broken arrow.

tection, using about 10 μg of cytoplasmic RNA isolated from uninfected *S. frugiperda* cells or cells at times after infection as indicated. For RNase protection analysis, specific RNA probes were synthesized in vitro from the cloned *Sty*I-*Eco*RI fragment, which was linearized with *Spe*I. Cytoplasmic RNA (10 μg) was annealed at 45°C overnight to the specific RNA probe (~10^5 Cerenkov cpm). After digestion with RNase A and RNase T_1, the protected hybrids were analyzed by electrophoresis on a 10% polyacrylamide gel containing 7 M urea (16).

For primer extension analysis, cytoplasmic RNA (10 μg) was annealed to an HE65-specific 30-base oligodeoxyribonucleotide primer (5'-TAGTGTAAATATGGTAGTCAGTG TCGCTGC-3'), an ME53-specific 28-base oligodeoxyribonucleotide primer (5'-TTGGCGCGTCAAAAATGTTATTCTC TCG-3'), or a p10-specific 30-base oligodeoxyribonucleotide primer (5'-GCGTCTAAAATTTGCGTCAAAACGTTAG GC-3'). Primers were labeled by [γ-^{32}P]ATP, using polynucleotide kinase. After hybridization at 50°C for 45 min, each of the primers was extended at 42°C for 2 h with 25 U of avian myeloblastosis virus reverse transcriptase (Stratagene) in the presence of 50 mM Tris HCl (pH 8.3), 20 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 5 U of RNase inhibitor (Sigma), and 500 μM each deoxynucleoside triphosphate. The primer extension products were denatured and analyzed on 6% polyacrylamide–7 M urea gels. As a marker, we determined the sequence of the construct pPE38-CAT109 (18) by the chain termination method (27), using the universal primer (Pharmacia).

Nucleotide sequence accession number. The nucleotide sequence accession number of the HE65 gene is X73577.

RESULTS AND DISCUSSION

By screening the cDNA library from poly(A)$^+$ RNA isolated 1 h p.i. with the cloned *Eco*RI Q fragment of AcNPV, we have identified three cDNA clones corresponding to an early 1.8-kb transcript. This early transcribed gene has a predicted molecular mass of 65 kDa and is flanked by the *hr*4left region and by an *Eco*RI site. Therefore, it was designated HE65; its location in comparison with the ME53, IE0/IE1, IEN, and PE38 genes is shown in Fig. 1.

Nucleotide sequence of the HE65 gene. By using synthetic primers, both strands of the HE65-specific cDNA clone 53 were sequenced. The nucleotide sequence of the genomic counterpart was determined from the *Eco*RI Q fragment. Figure 2 presents the sequence of the leftward-transcribed HE65 gene in the conventional orientation of the AcNPV genome (31). One major ORF of 1,662 nt with a coding capacity for 553 amino acids and a predicted molecular mass of 65 kDa has been identified. The deduced amino acid sequence showed no significant sequence homology to known polypeptides in the SWISSPROT data base. Our sequence data concerning the 3' and 5' regions of the HE65 gene confirmed those of the 3' end of the late transcribed p80 gene (21) and of part of the *hr*4left sequence (10).

Investigation of the two additional HE65-specific cDNA clones, 18 and 64, revealed a common 3' end of the cDNA clones 53, 18, and 64 following the single polyadenylation signal AATAA (Fig. 2). The precise mapping of the early 5' end by primer extension and RNase protection analyses reflected a transcriptional start site 11 to 12 and 14 to 15 nt upstream of the predicted ATG start codon (Fig. 2, 4, and 5). Sequences preceding the 5' end of HE65 share homology between 4 and 8 nt with other early AcNPV promoters (Fig. 3), including the GTAAG and CAGT motifs. These sequence elements have been suggested to represent characteristic features of late/very late or early promoters, respectively (for reviews, see references 12 and 23). In the case of the HE65 promoter, the early CAGT and the late GTAAG motifs are combined, but both elements do not represent the core of transcriptional initiation. The 5' ends of other early

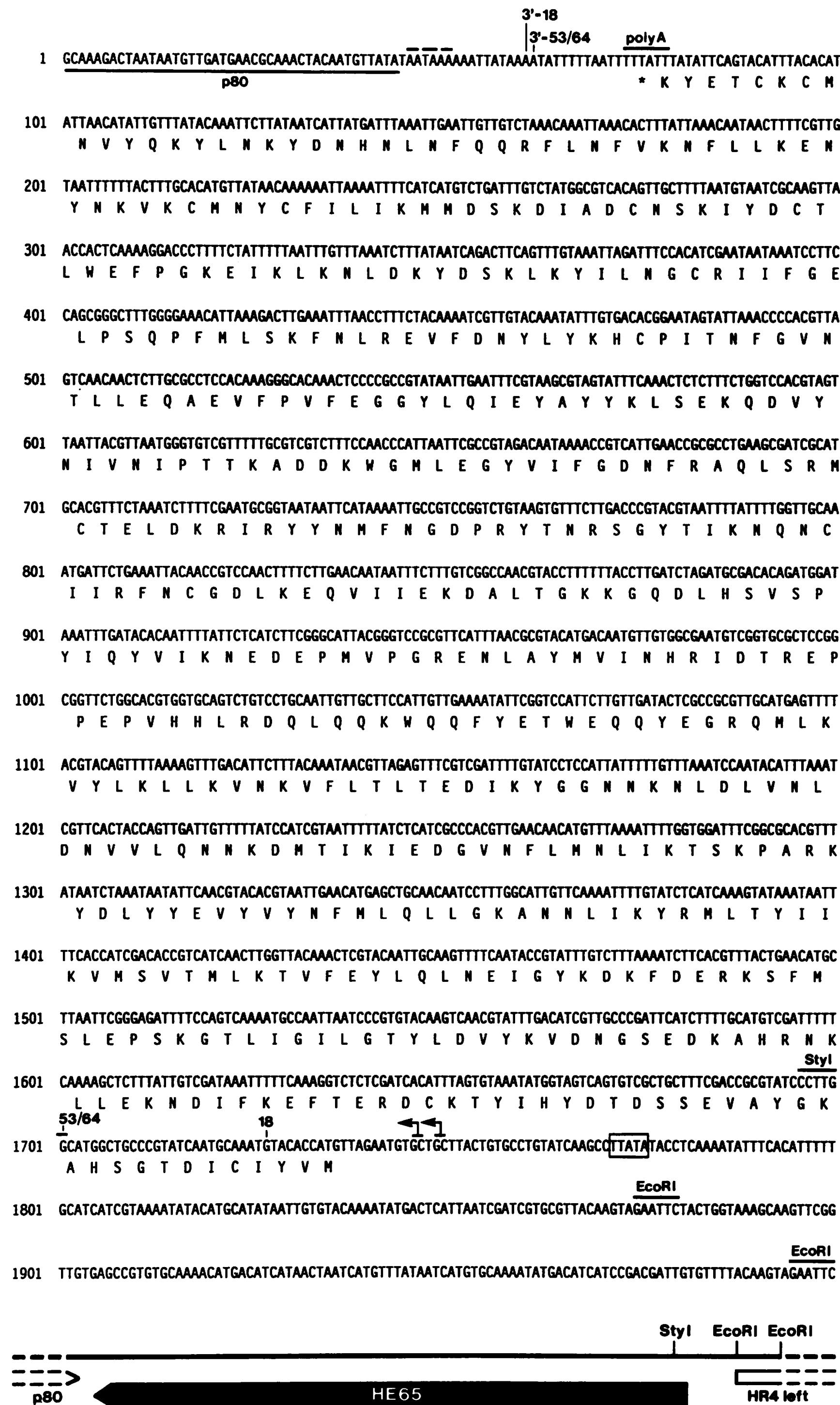

FIG. 2. Nucleotide sequence of the HE65 gene. The DNA sequence of 2,000 nt and the predicted amino acid sequence are shown in genomic orientation. Sequences overlapping the p80 gene are underlined. The potential polyadenylation signal of the p80 gene is indicated by a broken line; the corresponding signal of the HE65 gene is overlined. The 3′ and 5′ ends of three HE65-specific cDNA clones are shown by the numbers corresponding to cDNA clones 18, 53, and 64. Transcriptional initiation of the HE65 gene, which has been determined by RNase protection and primer extension analyses, is depicted by arrows. A possible TATA element is boxed.

genes such as CG30 (28), ETL (7), p143 (20), and 35K and 94K (8) exhibit no homology to the HE65 transcriptional start sequence.

The close spacing of the HE65 transcriptional and translational start sites of 10 nt is somewhat unusual. Untranslated leader sequences of the early genes PE38, IEN, ME53, IE1/IE0, 39K, and 35K are about 30 to 50 nt long (4, 5, 8, 11, 15, 19). In contrast, the early 94K gene has a leader of only

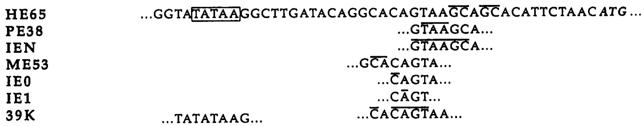

FIG. 3. HE65 promoter sequence homologies to other early AcNPV genes. Nucleotides homologous to HE65 sequences surrounding the transcriptional start are shown for the early promoters of genes PE38 (19), IEN (4), ME53 (16), IE1/IE0 (5, 15), and 39K (11). The transcriptional start sites are overlined. A potential TATA motif is boxed, and its identity with TATA sequences of the 39K gene is indicated.

11 nt (8). The functional significance of the different lengths of untranslated leader sequences is unknown.

Finally, the 5′ region of the HE65 gene carries a consensus TATA box element located 22 to 25 nt upstream of the HE65 transcriptional start. This element, which includes an ATAAG motif, is conserved over a stretch of 8 nt (Fig. 3) with the proximal TATA sequences of the 39K gene. The early transcription of the 39K gene is controlled by two TATA motifs each directing its own transcriptional start site (12).

Transcriptional analysis of the HE65 gene in comparison with the IEN, PE38, and ME53 genes. We have investigated the 5′ initiation site of the HE65 RNA in the course of infection by RNase protection and primer extension analyses. The results of the RNase protection experiments are shown in Fig. 4. Cytoplasmic RNA isolated from uninfected *S. frugiperda* cells or from cells at 1, 2, 4, 6, 12, 24, and 48 h p.i. were hybridized to a specific 283-nt RNA probe, which was synthesized in vitro from the cloned *Sty*I-*Eco*RI fragment. The protected fragments of 51 and 55 nt indicate the transcriptional initiation at 11 to 15 nt upstream of the predicted ATG start codon (Fig. 2). This transcriptional start site is weakly detectable at 2 h p.i., becoming more abundant at 4 to 48 h p.i. These findings have been confirmed by primer extension analyses. A synthetic oligodeoxyribonucleotide complementary to nt 53 to 91 downstream of the ATG start codon (Fig. 2) was annealed to cytoplasmic RNA and extended with reverse transcriptase. The extension products were analyzed on sequencing gels (Fig. 5). Extension products are visible from 4 to 45 h p.i. but hardly detectable at 2 h p.i. We have always identified four extension products which correspond to GC pairs (indicated by arrows in Fig. 5) at nt 11/12 and 14/15, respectively, upstream of the translational start codon. Further extension products have been observed at 12 and 24 h p.i. The product of about 126 nt, which corresponds roughly to the protected fragments of 79 and 82 nt in RNase protection analysis (Fig. 4), reflects a weak transcriptional initiation at the consensus TATA sequences. An extension product of about 175 nt might also be due to a transcriptional start site at the nucleotides TAT, but this finding is not consistent with the data of the RNase protection analyses. The protected fragment of about 183 nt

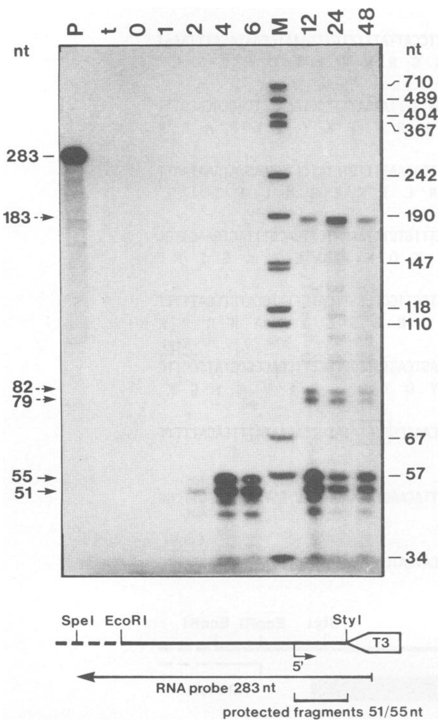

FIG. 4. RNase protection analysis of HE65 transcription in the course of infection. Cytoplasmic RNAs (10 μg) isolated from uninfected *S. frugiperda* cells (lane 0) or from cells at 1, 2, 4, 6, 12, 24, and 48 h p.i. (lanes 1, 2, 4, 6, 12, 24, and 48) or tRNA (lane t) were hybridized to the RNA probe of 283 nt. The hybrids were analyzed on a 10% polyacrylamide sequencing gel, and the autoradiogram was exposed overnight. The major protected hybrids of 51 and 55 nt are indicated by arrows, and additional hybrids, visible during the late phase of infection, are shown by broken arrows. The size of the RNA probe (lane P) is indicated on the left, and positions of DNA size markers (lane M) are shown on the right.

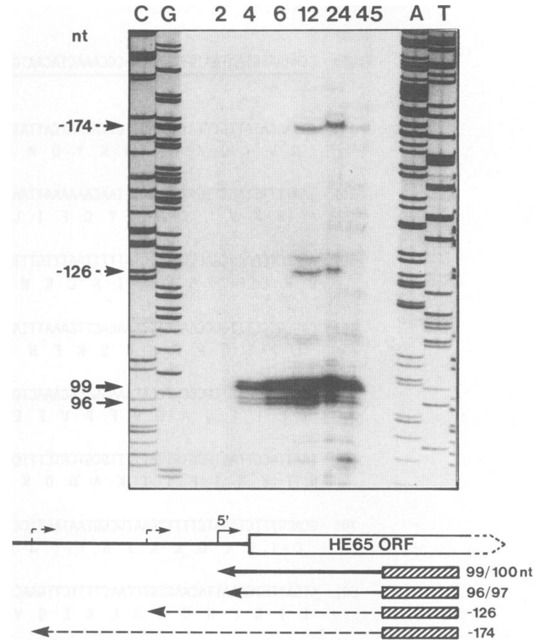

FIG. 5. Primer extension analysis of HE65 transcription in the course of infection. Cytoplasmic RNAs (10 μg) prepared at 2, 4, 6, 12, 24, and 45 h p.i. (lanes 2, 4, 6, 12, 24, and 45) were hybridized to a 30-mer oligodeoxyribonucleotide primer. The extended products were analyzed along with the sequencing ladder of pPE38-CAT109 (lanes C, G, A, and T) on a 6% polyacrylamide sequencing gel. The time of exposure for the autoradiogram was 2 days. The major extended products of 99/100 and 96/97 nt are depicted by arrows; further extension products of about 126 and 174 nt as well as the localization of the corresponding start sites are indicated by broken arrows.

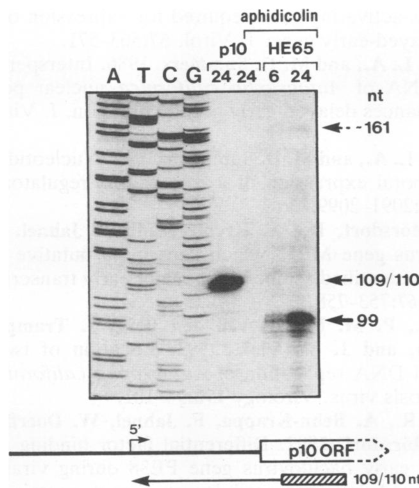

FIG. 6. Primer extension analysis of the effect of aphidicolin on HE65 transcription. Cytoplasmic RNA (2 μg) prepared at 24 h p.i. (lane 24) and RNA (20 μg) prepared from aphidicolin-treated cells at 24 h p.i. (lane 24, aphidicolin) were hybridized to the p10-specific oligodeoxyribonucleotide primer. Cytoplasmic RNA preparations (20 μg) from aphidicolin-treated cells at 6 h p.i. (lane 6, aphidicolin) or 24 h p.i. (lane 24, aphidicolin) were hybridized to the HE65-specific oligodeoxyribonucleotide primer. Further details are as described for Fig. 5.

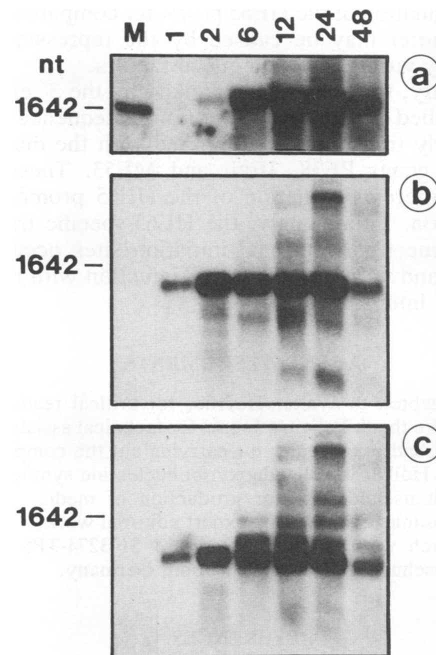

FIG. 7. Northern blot analysis of HE65-specific RNAs in comparison with IEN and PE38 transcripts. Poly(A)$^+$ RNA was isolated 1, 2, 6, 12, 24, and 48 h p.i. (lanes 1, 2, 6, 12, 24, and 48), and 5 μg of each preparation was analyzed on a 1.4% agarose gel containing 2.2 M formaldehyde. After transfer to a nitrocellulose filter, the RNA was hybridized to the HE65-specific cDNA clone 53 (a) and rehybridized to an IEN-specific (b) or PE38-specific (c) cDNA clone. Positions of DNA size markers (lane M) are indicated on the left.

(Fig. 4) corresponds to protection of the RNA probe, suggesting transcriptional initiation further upstream of the *Eco*RI site in the *hr*4left region (Fig. 2). These weak start sites in the late phases of infection have not been analyzed in further detail.

Since early viral genes are proposed to be transcribed independently of viral replication (26), we have studied the effect of the AcNPV DNA replication inhibitor aphidicolin on the level of HE65-specific transcripts in the course of infection. As a control, the inhibitory effect of aphidicolin on late viral transcription has been demonstrated for the late p10 gene, confirming previous data (26), (Fig. 6). In contrast, HE65 transcription is not blocked in aphidicolin-treated *S. frugiperda* cells at 6 and 24 h p.i. (Fig. 6), emphasizing the early transcription of the HE65 gene. The weak start sites detected in untreated cells at 24 h p.i. (Fig. 5) are probably late transcriptional start sites, since they are influenced by the aphidicolin treatment (Fig. 6).

To investigate the temporal regulation of HE65 transcripts in comparison with the IEN and PE38 transcription, poly(A)$^+$-selected RNA prepared from AcNPV-infected *S. frugiperda* cells at various times p.i. was analyzed on Northern blots. After hybridization to the HE65-specific cDNA clone 53, a transcript of about 1,800 nt was observed at 2, 6, 12, 24, and 48 h p.i. (Fig. 7a). Its size is consistent with transcription of the predicted ORF of 1,662 nt and a poly(A) tail of about 150 nt. Several weakly expressed RNAs overlap the HE65 gene at 12 and 24 h p.i.; two RNA size classes are smaller and three transcripts are larger than the major HE65 RNA of 1,800 nt (data not shown). The transcriptional pattern of IEN and PE38 RNAs, which was previously described in detail (19), differs in the abundance of early transcripts compared with the temporal appearance of HE65-specific RNAs (Fig. 7b and c). In contrast to the HE65 RNA, the IEN transcript of 1,500 nt and the PE38 transcript of 1,300 nt are clearly detectable at 1 h p.i. A comparable intensity of HE65 transcription is apparent only at 2 h p.i., indicating a delay in transcriptional activity of about 1 h.

The retarded transcriptional initiation of the HE65 gene in comparison with the early ME53 transcription was investigated by primer extension analyses. As indicated in Fig. 8, the early ME53 transcript initiates at a CA pair preceding the CAGT motif (Fig. 3) and can be identified 2 h p.i. The low abundance of early HE65 transcripts at 2 h p.i. indicating a

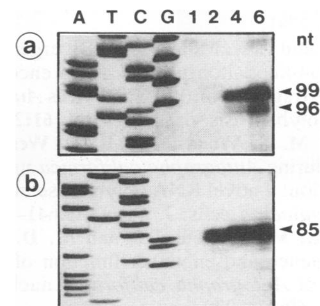

FIG. 8. Comparison of early HE65 and ME53 transcriptional initiation by primer extension analysis. Cytoplasmic RNAs (10 μg) isolated 1, 2, 4, and 6 h p.i. (lanes 1, 2, 4, and 6) were hybridized to a 30-mer oligodeoxyribonucleotide primer homologous to the HE65 ORF (a) or to a 28-mer oligodeoxyribonucleotide primer homologous to the ME53 ORF (b) and analyzed as described for Fig. 5. The extended products (arrowheads) were separated on the same 6% polyacrylamide sequencing gel. The sequencing ladder of pPE38-CAT109 (lanes A, T, C, and G) was coelectrophoresed.

different regulation of the HE65 promoter compared with the ME53 promoter may be caused by the repression and/or activation by virus- or host-induced factors.

In summary, the HE65 gene flanked by the 3′ end of the late transcribed p80 gene and the *hr*4left sequence shows a delay in early transcription compared with the major early transcribed genes PE38, IEN, and ME53. These results suggest a different regulation of the HE65 promoter early after infection. Interestingly, the HE65-specific transcripts with the same transcriptional initiation sites occur during early, late, and very late phases of infection with no significant loss in intensity.

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