Physical Mapping of Temporally Regulated, Overlapping Transcripts in the Region of the 10K Protein Gene in *Autographa californica* Nuclear Polyhedrosis Virus

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We investigated transcriptional activity in the region of a gene for a major late protein (10 kilodaltons) of *Autographa californica* nuclear polyhedrosis virus. This 10K protein gene spans an *Hind*III cleavage site, with the 5' end of the gene located in the *Hind*III-Q fragment and the 3' end in the *Hind*III-P fragment. Northern blot analysis showed that there were at least four transcripts mapping in this region, two that are present maximally at 12 h (1,500 and 1,100 bases in length) and two that are present at 24 h (750 and 2,500 bases in length). Northern blot analysis also suggested that these transcripts overlap. S1 mapping identified the precise positions of the transcripts and confirmed the overlap. The 1,500- and 1,100-base transcripts share a common 5' end, about 1,056 bases upstream from the *Hind*III-P-Q boundary; the 750- and 2,500-base transcripts share a common 5' end, about 303 bases upstream from the *Hind*III-P-Q boundary. The 1,100-base transcript terminates in the Q fragment, about 234 bases from the *Hind*III-P-Q boundary. The other three transcripts all end in the P fragment. Both the 1,500- and the 750-base transcripts terminate approximately 240 bases from the *Hind*III-P-Q boundary. The 2,500-base transcript exhibits 3'-end heterogeneity, ending around 2,046 bases from the *Hind*III-P-Q boundary. Therefore, three different transcripts (1,500, 750, and 2,500 bases in length) contain the complete 10K protein coding region. A fourth transcript (1,100 bases in length) overlaps the 750-base 10K protein gene transcript by 70 bases.

Autographa californica nuclear polyhedrosis virus (AcMNPV) replicates in the nucleus of host insect cells. The virions are typically occluded, although the nonoccluded form is thought to be responsible for cell-to-cell transmission within the infected insect larvae (12). The latticelike structure that surrounds the virions to form the inclusion body consists of crystallized polyhedrin, a protein of molecular weight 29,000 (13).

In addition to polyhedrin, approximately 40 other virus encoded proteins have been found (5, 7, 20). These proteins appear at successive stages after viral infection, indicating temporal control of viral protein synthesis (5, 20, 31). At least some of this temporal control is exerted at the level of transcription, as demonstrated by the presence of different RNA messages at different times during the course of infection (10, 11, 24, 28). Some viral transcripts are synthesized far more abundantly than others (23, 28). Therefore, at any one time, the virus regulates not only which gene or genes are transcribed, but also how actively each gene is transcribed.

Viral messages, like other eucaryotic mRNAs, are both capped (22) and polyadenylated (27). However, introns, a prominent feature of genes for most eucaryotic messages, appear to be absent from AcMNPV genes (15).

As viral transcripts have been mapped to the AcMNPV genome, a complex pattern of transcription has emerged. Although all viral RNAs examined thus far have been transcribed from the same DNA strand, the AcMNPV genes do not seem to be clustered according to the time of their activity, as are the T7 phage genes, for instance (6). Different AcMNPV transcripts, synthesized at the same time during the course of infection, mapped to genes that were scattered throughout the genome (1, 9, 26-28).

Recently, further transcriptional complexity has been discovered in the form of overlapping transcripts (11, 16). Some of these overlapping transcripts share a common 5' end, others have a common 3' end. It has been hypothesized that this type of transcriptional organization may function to regulate the temporal expression of genes in some cases (11).

There are two genes that are very active late in the infection cycle (5). One codes for polyhedrin; the other codes for a protein of 10,000 daltons (10K protein) whose function is not known. We investigated transcriptional activity in the region of the 10K protein gene and present those results in this paper. Four overlapping transcripts mapped to this region. Northern blots and S1 mapping were used to identify the overlapping segments, to map the 5' and 3' ends of each transcript to the genome, to show the temporal regulation of each transcript, and to search for clues as to how that regulation might be accomplished.

MATERIALS AND METHODS

Biochemicals. S1 nuclease and nucleic acid chromatography system (NACS) columns were purchased from Bethesda Research Laboratories, Inc. T4 polynucleotide kinase was obtained from Promega Biotec; DNA polymerase I was from Boehringer Mannheim Corp.; and Klenow fragment of *Escherichia coli* DNA polymerase I was from International Biotechnologies, Inc. Restriction endonucleases were purchased from both Bethesda Research Laboratories and International Biotechnologies. Technical-grade CsCl was obtained from Kawecky Berylo Industries, Inc., and low-melting-point (Seaplaque) agarose was from the FMC Corp., Rockland, Maine. Radioisotopes, α - 32 P-dinucleoside triphosphates, and [γ - 32 P]ATP, were purchased from New England Nuclear Corp. Methylmercuric hydroxide was obtained from Alfa Products, Danvers, Mass.

Preparation of DNA probes for Northern blot analysis.

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AcMNPV DNA fragments, produced by digestion with various restriction enzymes, were cloned in pBR322. Large-scale preparations of plasmid DNA were purified either by ultracentrifugation in CsCl gradients or by passage over an NACS column. To produce subfragments of DNA for probes, cloned fragments of DNA were cleaved with one or more restriction endonucleases (*Hind*III, *Eco*RI, *Xho*I, *Bgl*II, or *Sst*I). Individual fragments of interest were separated on the basis of size by electrophoresis in and elution from low-melting-point agarose (17). The purified AcMNPV subfragments were labeled by nick translation (19).

Preparation of RNA from NPV infected *Spodoptera frugiperda* cells. *S. frugiperda* cells were infected with nonoccluded virions (10 50% tissue culture infectious doses per cell) of AcMNPV. At 2, 6, 12, 24, and 40 h postinfection (p.i.), polysomal RNA was isolated from infected cell cultures essentially by the method of Efstratiadis and Kafatos (8). Polyadenylated [poly(A)⁺] RNA was then isolated from total polysomal RNA by oligo(dT)-cellulose chromatography.

RNA fractionation via methylmercury gel electrophoresis and transfer to aminothiophenol paper. Poly(A)⁺ RNA was size fractionated on denaturing agarose gels containing 5 mM methylmercury hydroxide (CH₃HgOH) as described by Bailey and Davidson (3). Aminothiophenol paper was prepared by the method of Seed (25). The methylmercury gel was prepared for transfer, and the paper was activated by the method of Alwine et al. (2). The gel was also blotted to the activated paper by the method of Alwine et al. (2).

Northern blot hybridization. The RNA blots were soaked in 10 ml of prehybridization solution (5X SSC [1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.04 M Na₂HPO₄ [pH 6.5], 0.2% sodium dodecyl sulfate, 2X Denhardt solution, 10 µg of denatured calf thymus DNA per ml, 0.1 g of glycine per ml) and incubated at 42°C for 15 to 18 h. DNA probes were nick translated (19) in the presence of [α -³²P]dATP to a specific activity of about 10⁷ cpm/µg; then they were denatured by heating and were added to the hybridization solution. The hybridization solution was the same as the prehybridization solution, except that the glycine was omitted. After hybridization at 42°C for 15 to 18 h, the blots were washed two times in 2X SSC-0.5% sodium dodecyl sulfate for 15 min each and two times in 0.1X SSC for 30 min each. All washes were done at 42°C. The blots were dried and autoradiographed with DuPont Cronex X-ray film.

Preparation of probes for S1 mapping. Two different strategies were used to label the DNA probes at the desired site. In one, the insert was cleaved from the pBR322 vector and was then ³²P labeled at the 5' end with T4 polynucleotide kinase (21) or at the 3' end with the Klenow fragment of *E. coli* DNA polymerase I (17). Labeled DNA was then purified from unincorporated mononucleotides by Sephadex G-100 chromatography on a column that had been prerun with 50 µg of tRNA. To obtain probes that were labeled at only one end, the insert was then cleaved asymmetrically with a second restriction enzyme, and the desired portion of labeled insert was isolated by electrophoresis on a low-melting-point agarose gel.

In the other strategy, the insert DNA was labeled at an internal cleavage site by first cleaving the cloned DNA at the site to be labeled. The DNA was then labeled and cleaved with a second restriction enzyme to liberate the insert DNA from the plasmid DNA. The desired portion of labeled insert was isolated and purified as detailed above.

RNA purification from methylmercury hydroxide agarose

gels. Poly(A)⁺ RNA was size fractionated in methylmercury hydroxide gels as described above, except that low-melting-point agarose was used. The methods used to recover the RNA from the agarose gel slices were essentially those of Maniatis et al. (18).

RNA-DNA hybridization and S1 mapping. Approximately 1,500 cpm (Cerenkov counts) of end-labeled DNA probe was hybridized to 30 µg of total cytoplasmic RNA or 1 µg of poly(A)⁺ RNA as described by Weaver and Weissmann (29) and Berk and Sharp (4). The hybridization reaction was then incubated at 42°C for 14 to 18 h. The S1 nuclease reaction and polyacrylamide gel electrophoresis were done by the method of Weaver and Weissmann (29). The gels were autoradiographed with Kodak X-Omat AR film or DuPont Cronex X-ray film.

RESULTS

Northern blot analysis. To identify transcripts in the 10K protein region, Northern blots were probed with labeled subfragments of *Hind*III-Q and *Hind*III-P restriction fragments. Figure 1a shows subfragments from *Hind*III-Q (400-base-pair [bp] *Eco*RI-*Sst*I fragment, 670-bp *Sst*I-*Hind*III fragment) and from *Hind*III-P (1,380-bp *Hind*III-*Eco*RI fragment, 530-bp *Eco*RI-*Xho*I fragment, 290-bp *Xho*I-*Hind*III fragment) that were gel purified and nick translated for use as probes.

Polysomal poly(A)⁺ RNA for the blots was obtained from *S. frugiperda* cells at 2, 6, 12, 24, and 40 h after infection with AcMNPV. The 400-bp *Eco*RI-*Sst*I probe from the *Hind*III-Q fragment hybridized to four different-sized RNAs (Fig. 1b). Two, 1,500 and 1,100 bases long, first appeared at 6 h p.i. and were present in greatest amounts at 12 h p.i. At 24 h p.i., a 2,500-base transcript and a transcript of ~750 bases were seen. According to our S1 mapping data, discussed below, neither the 2,500-base transcript nor the 750-base transcript extended into the 400-bp *Eco*RI-*Sst*I region of *Hind*III-Q; the 5' ends of these transcripts mapped to the right of the 400-bp region. The most likely explanation for the discrepancy is that our 400-bp probe in the Northern blot experiment was contaminated with *Xho*I-*Hind*III-cleaved (~500-bp) DNA from the *Hind*III-Q fragment.

The 670-bp *Sst*I-*Hind*III fragment of Q (Fig. 1c) should hybridize to the 5' end of the transcript for the 10K protein (see Fig. 3e). An intense band of ~750 bases was seen in the lane containing RNA from 24 h p.i. The abundance and size of the message, in addition to its late appearance, all indicated that this band identified the message for the 10K protein. Another transcript that was maximally present at 24 h was approximately 2,500 bases in length. Two additional transcripts of 1,500 and 1,100 bases first appeared at 6 h and were most abundant at 12 h. Considering their size, and the timing of their appearance, it seems likely that they are the same 1,500- and 1,100-base transcripts that hybridized to the upstream 400-bp probe (Fig. 1b).

Of the four transcripts hybridizing to the 670-bp probe, the 750-base, 10K protein message was synthesized in the greatest amount, and the 1,100-base transcript was synthesized in the least amount. The 2,500- and 1,500-base transcripts were produced in moderate amounts.

The 1,380-bp probe (*Hind*III-*Eco*RI) from the left end of the P fragment hybridized to three transcripts (Fig. 1d). These transcripts (750, 1,500, and 2,500 bases in length), due to their size and timing of synthesis, appeared to be the same as those that hybridized to the 670-bp subfragment of the Q fragment as well. Probes from the far right-hand side of *Hind*III-P hybridized to two transcripts, ~2,500 and ~800

FIG. 1. Northern blot analysis of transcripts derived from the AcMNPV *HindIII*-Q and -P fragments. Polysomal, poly(A)⁺ RNA was isolated at 2, 6, 12, 24, and 40 h p.i., fractionated on a methylmercury hydroxide gel, and blotted to aminothiophenol paper. The blots were hybridized to nick-translated probes. (a) Location of the *HindIII*-P and -Q subfragments used to probe the Northern blots. (b through f) Northern blots with subfragments of *HindIII*-P and -Q. Markers are *HaeIII*-cleaved ϕ X174 and *HindIII*-cleaved λ DNAs.

bases in length (Fig. 1e and f). Both transcripts appeared maximally at 24 h, and the 2,500-base transcript was in greater abundance than was the 800-base transcript.

The hybridization to the *HindIII*-P probes shows that the 2,500-, 1,500-, and 750-base transcripts extend into the left-hand part of *HindIII*-P. The 2,500-base transcript hybridized to all *HindIII*-P probes, suggesting that it extends to within 300 bases of the right end of *HindIII*-P. The 800-base transcript that hybridized to the right-hand probes could be the same as the 750-base transcript if there is an intervening

sequence in the 3' end of the gene. However, in light of Lübbert and Doerfler's data (15), it seems more plausible that the 800-base transcript is independent of the 750-base transcript.

S1 mapping with size-fractionated poly(A)⁺ RNA. Polysomal poly(A)⁺ RNA was isolated from *S. frugiperda* cells 12 h p.i. and fractionated on a low-melting-point agarose gel that contained the denaturant CH₃HgOH. The RNA lanes were sliced into 0.5-cm strips perpendicular to the direction of migration. RNA was isolated from each strip

FIG. 2. S1 nuclease mapping with size-fractionated poly(A)⁺ RNA. (a) A DNA probe was made by cleaving the circular DNA from an AcMNPV *HindIII*-Q clone with *Bgl*II and then labeling the linearized DNA at its 5' ends with T4 polynucleotide kinase. A second cleavage (with *HindIII*) liberated a 2,120-bp subfragment, 5' end labeled at the *Bgl*II site. This 2,120-bp fragment was purified from a low-melting-point agarose gel and used in S1 mapping experiments as described below. (b) Lanes 1 and 2 contain *Hae*III-cleaved ϕ X174 and *Hae*-III cleaved pBR322, respectively. The 5'-labeled probe (a) was hybridized to size-separated RNA (12 h p.i.) of the following sizes, S₁ nuclease treated, and subjected to polyacrylamide gel electrophoresis and autoradiography. Lanes: 3, ~2,500 bases; 4, ~1,500 bases; 5, ~1,100 bases; 6, ~750 bases; 7, total poly(A)⁺ RNA from 12 h p.i.; 8, no RNA.

and hybridized to a 5' end-labeled 2,120-bp probe from the *HindIII*-Q fragment (Fig. 2a). After treatment with S1 nuclease, polyacrylamide gel electrophoresis, and autoradiography, bands representing regions of DNA probe protected from S1 digestion were seen. The fraction containing transcripts approximately 2,500 bases in length protected 223 bp of probe (Fig. 2b, lane 3). Transcription products approximately 1,500 bases long protected 976 bp of probe (Fig. 2b, lane 4). No labeled DNA probe was protected upon hybridization to the fraction of RNA containing transcripts 1,100 bases in length (Fig. 2b, lane 5). Although a 1,100-base transcript mapped within the 2,120-bp portion of fragment Q (data presented below), the 1,100-base transcript did not

span the *Bgl*II site and therefore did not protect the label. RNA of approximately 750 bases again protected 223 bp of the probe (Fig. 2b, lane 6). That at least three different sizes of RNA mapped to this same region of DNA verified what the Northern blot analysis had suggested: there are overlapping transcripts synthesized from this region of the DNA. To verify that the bands in lanes 3, 4, and 6 of Fig. 2b were not artifacts of the S1 nuclease reaction, the probe alone (without RNA) was treated with S1 (Fig. 2b, lane 8). When the poly(A)⁺ RNA from 12 h p.i. was not size fractionated, S1 mapping yielded two protected fragments (Fig. 2b, lane 7), the same lengths (976 and 223 bp) as those seen with the size-selected RNA (Fig. 2b, lanes 3, 4, and 6). These data suggest that the 750- and 2,500-base transcripts share a common 5' end that maps about 223 bp to the left of the *Bgl*II site in *HindIII*-Q. The 5' end of the 1,500-base transcript maps further upstream, about 976 bp to the left of the *Bgl*II site.

Mapping the 10K protein transcript. To confirm the location of the 5' end of the 750-base transcript coding for the 10K protein, pBR322 containing the *HindIII*-Q fragment was cleaved with *HindIII*, ³²P labeled at the 5' ends, and then restricted with *Sst*I (Fig. 3a). The labeled 670-bp *Sst*I-*HindIII* fragment was gel isolated and hybridized to total cytoplasmic RNA from 40 h p.i. Our S1 mapping results were in agreement with those from other laboratories (14, 16); a protected fragment of 303 bases was seen (Fig. 3b, lane 2). There was also protection of the entire 670-bp probe, suggesting that at least one other transcript overlapped the 5' end of the 750-base transcript and extended upstream at least as far as the *Sst*I cleavage site. The protection of the whole probe is not due to probe annealing, because no protection was seen in the absence of RNA (Fig. 2b, lane 8).

Artifacts of the S1 nuclease reaction (seen as minor bands; Fig. 3b, lane 2) are caused by breathing of the hybrid. These minor bands decreased in intensity when the S1 reaction was incubated at a lower temperature (data not shown). In addition, the pattern of these artifactual bands changed when the DNA probe was hybridized to RNA isolated from gels containing CH₃HgOH (data not shown). Since CH₃HgOH is a denaturing agent, any residual CH₃HgOH bound to the RNA causes local denaturation in an RNA-DNA hybrid, changing the breathing pattern of the hybrid and allowing S1 to cleave at different sites.

The 3' end of the 750-base transcript was mapped with a probe from the *HindIII*-P fragment (Fig. 3c). A recombinant plasmid containing *HindIII*-P was cleaved with *HindIII*, and the recessed 3' ends at the *HindIII* sites were filled in with ³²P-labeled nucleotides. The labeled fragment was then cleaved with *Xho*I, and the 1,910-bp piece was used in S1 mapping with size-selected RNA (~750 bases) (Fig. 3d, lane 4). A total of 240 bases of the probe were protected from S1 digestion, placing the 3' end of the 750-base transcript 240 bases downstream of the *HindIII*-P-Q boundary (Fig. 3e). A band near the top of the gel represents reannealed probe. The 872-nucleotide band (Fig. 3d, lane 4) is apparently an artifact of the S1 nuclease reaction, since it was also present when the probe alone was treated with S1 nuclease (Fig. 3d, lane 3).

Mapping the 2,500-base transcript. By hybridizing size-selected poly(A)⁺ RNA from 24 h p.i. to the probe diagrammed in Fig. 2a, we confirmed that the 5' end of a 2,500-base transcript mapped to the same genomic location as the 5' end of the 750-base 10K protein transcript (Fig. 4a, lanes 1 and 2). Therefore, the 5' end of the 2,500-base transcript, like the 750-base transcript for the 10K protein, is

FIG. 3. Mapping the 10K protein transcript. (a) A 5' end-labeled 670-bp probe was made from the *Hind*III-Q fragment by labeling the *Hind*III-cleaved 5' ends with ³²P, restricting with *Sst*I, and isolating the 670-bp subfragment. (b) The isolated 670-bp fragment was used as a probe for S1 mapping with total late RNA (40 h p.i.) (lane 2). Lanes 1 and 3 contained pBR322 and ϕ X174 markers, respectively. (c) The *Hind*III-P fragment was 3' end labeled at the *Hind*III sites and then cleaved with *Xho*I. A 1,910-bp, *Hind*III-*Xho*I subfragment was isolated and used as a probe for S1 mapping. (d) Lanes: 1, 2, 5, and 6, markers; 3, S1-treated, 1,910-bp DNA probe alone; 4, S1 mapping with 1,910-bp probe and size-selected 750-base poly(A)⁺ RNA. (e) Location of the 750-base 10K protein transcript with respect to the *Hind*III-Q and -P fragments.

located in fragment Q, 303 bases upstream from the *Hind*III-Q-P boundary (Fig. 4d). This confirms the inference Lübbert and Doerfler drew from their S1 mapping experiments with different probes (16).

The 3' end of the 2,500-base transcript is located near the far right-hand side of the P fragment (Fig. 4d). A 290-bp, 3' end-labeled probe (Fig. 4b) was partially protected by hybridization to RNA from 24 h p.i. Three different bands representing protected fragments of 290, 275, and 132 to 140 bp were seen (Fig. 4c, lane 4). The 290-bp band may be reannealed probe; however, no reannealed probe was seen

in the absence of RNA (Fig. 4c, lane 3), suggesting that some transcription may continue through the whole 290-bp probe. The other bands apparently result from S1-treated RNA-DNA hybrids, since S1 treatment of probe alone (Fig. 4c, lane 3) gave no detectable bands. When size-selected transcripts 2,500 bases in length were hybridized to the 290-bp probe (Fig. 4c, lane 5), a very weak band representing protection of 275 bases of probe and a wide band showing protection of probe fragments of specific lengths ranging from 132 to 140 bp were seen (Fig. 4c, lane 5). The 2,500-base transcript appears to have two different 3' ends,

FIG. 4. Mapping the 2,500-base transcript. (a) A DNA probe, 5' labeled at the *Bgl*II site, was used to S1 map the 5' end of the 2,500-base transcript. Lanes: 1, mapping with size-selected 2,500-base RNA; 2, mapping with size-selected 750-base RNA; 3, S1-treated probe, without RNA; 4 and 5, markers. (b) A 290-bp subfragment from the far right-hand side of P was 3' labeled at the *Xho*I site, purified, and used as a probe to identify the 3' end of the 2,500-base transcript. (c) Lanes 1, 2, 6, and 7 contained markers. S₁ mapping experiments were carried out with the following (lanes): 3, no RNA; 4, poly(A)⁺ RNA 24 h p.i.; 5, size-selected 2,500-base RNA from 24 h p.i. (d) Location of the 2,500-base transcript with respect to the *Hind*III-Q and -P fragments.

and one of those ends exhibits microheterogeneity. This maps the 3' end of the 2,500-base transcript around 2,046 bases downstream from the *Hind*III-P-Q boundary (Fig. 4d).

Mapping the 1,500-base transcript. The 5' end of the 1,500-base transcript was located 1,056 bases from the *Hind*III-P-Q boundary (Fig. 5b), as determined by the S₁ mapping described above (Fig. 2a; Fig. 2b, lane 4). The 3' end of the 1,500-base transcript mapped to the same location as the 3' end of the 750-base transcript, 240 bases from the *Hind*III-P-Q boundary (Fig. 5b). To eliminate 750-base transcripts from this S₁ mapping experiment, RNA isolated

at 6 h p.i. was used. As shown above (Fig. 1c), this RNA contains the 1,500-base transcript, but not the 750-base transcript. This RNA from 6 h p.i. gave a protected fragment exactly the same size (240 bases) as that seen when RNA from 24 h was used (Fig. 5a, lanes 4 and 5).

Mapping the 1,100-base transcript. A 5' end-labeled DNA probe made from the 1,735-bp *Hind*III-*Xho*I fragment of Q (Fig. 6a) was hybridized to size-selected poly(A)⁺ RNA from 12 h p.i. Fractions containing transcripts 1,500 bases in length (Fig. 6b, lanes 1 and 2) protected 588 nucleotides of the probe. RNA from the fraction containing transcripts of

1,100 bases also protected 588 nucleotides of the probe (Fig. 6b, lane 4). Transcripts of intermediate size, between 1,100 and 1,500 bases, did not protect the label (Fig. 6b, lane 3). Therefore, the 1,500- and 1,100-base transcripts share a common 5' end, about 1,056 bases from the *Hind*III-P-Q boundary.

The 3' end of the 1,100-base transcript was mapped to about 231 bp downstream from the *Xho*I site in the Q fragment (Fig. 6e). A 465-bp *Xho*I-*Hind*III segment of DNA, 3' labeled at the *Xho*I site was used to probe poly(A)⁺ RNA isolated 12 h p.i. (Fig. 6c). A total of 231 bp were protected from S1 nuclease digestion (Fig. 6d, lane 3). Reannealing of probe and hybridization to the 1,500-base transcript both would protect the entire probe from S1 nuclease and could have contributed to the 465-bp band in Fig. 6d, lane 3.

Location of four overlapping transcription products on the AcMNPV genomic map. Figure 7 summarizes the mapping of the four overlapping transcripts. The two upstream transcripts (1,100 and 1,500 bases) were most abundant around 12 h p.i. The two "downstream" transcripts (750 and 2,500 bases) were most prevalent about 24 h p.i. All four transcripts mapping in the region of the 10K protein gene were transcribed from the same DNA strand. We labeled DNA probes at the *Hind*III-P-Q boundary and at the *Xho*I site within the *Hind*III-Q fragment (Fig. 7) such that transcription products from the opposite strand would protect a portion of labeled probe during subsequent S1 nuclease

treatment. No protection was observed (data not shown); therefore, we concluded that the opposite strand was not transcribed.

DISCUSSION

AcMNPV provides an attractive system for investigating temporal regulation of eucaryotic genes. To learn more about such regulation in AcMNPV, we investigated transcription products from the region of the 10K protein gene. Figure 7 summarizes the results of our mapping of four different transcripts in the region of the 10K protein gene. These overlapping transcripts comprise two sets: (i) a late, γ -phase (11) pair of transcripts (1,100 and 1,500 bases) that are most abundant 12 h p.i. and whose 5' ends map about 1,056 bp upstream from the *Hind*III-P-Q boundary; (ii) a very late, δ -phase (11) pair of transcripts (750 and 2,500 bases) that are most abundant 24 h p.i. and whose 5' ends map about 303 bp upstream from the *Hind*III-P-Q boundary (Fig. 7).

Overlapping transcripts help explain the observation (15) that AcMNPV produces many more transcripts than proteins. Such transcripts have been found in other regions of the AcMNPV genome. One set of four overlapping transcripts, mapping between 32.6 and 41.0 AcMNPV map units, appeared to share a common 3' end (16). Another group of transcripts has been reported in the *Hind*III-K region, adjacent to the *Hind*III-Q fragment (11, 15). The overlapping transcripts of this set terminate at a common 3' end that has been mapped within the *Hind*III-Q fragment (11).

Further information about the overlapping transcripts synthesized from the region of the 10K protein gene in AcMNPV has come from sequence data. Our preliminary results indicate that there is an open reading frame, long enough to code for a polypeptide of approximately 240 amino acids, upstream from the 5' end of the 750-base transcript (A. F. Liu, J. C. Qin, and R. F. Weaver, manuscript in preparation). This open reading frame lies within both the 1,100- and the 1,500-base transcripts; consequently, the 1,500-base transcript contains coding sequences for two polypeptides, the 10K protein and the product of the upstream open reading frame.

Examination of the published sequence for the 10K protein gene (14, 16) showed possible polyadenylation signals for three of the four overlapping transcripts. The 3' end of the 1,100-base transcript is about 231 bases downstream from the *Xho*I site in the *Hind*III-Q fragment (Fig. 6e). This means that the 1,100-base transcript ends near the nucleotide immediately 5' of the initiating methionine codon of the 10K protein gene. The motif ATTAAA, a variation of the polyadenylation consensus sequence (30), is located 34 nucleotides upstream from the end of the 1,100-base transcript. Relative to the 10K protein gene, this polyadenylation signal is located within the untranslated leader region.

Sequence data (14) are also available for the two transcripts (750 and 1,500 bases long) that share a common 3' end (Fig. 7). A polyadenylation consensus sequence, AATAAA, is found in the *Hind*III-P fragment, 215 nucleotides from the *Hind*III-P-Q boundary. The transcripts end 20 bases 3' of this polyadenylation signal, as predicted by Kuzio et al. (14). This polyadenylation signal matches the conserved AATAAA consensus sequence (30), and the two transcripts (1,500 and 750 bases) formed by its utilization are more abundant than the other two, which use alternative signals. At present, there is no sequence information available for the region of the *Hind*III-P fragment to which the 3' ends of the 2,500-base transcript have been mapped.

FIG. 5. Locating the 3' end of the 1,500-base transcript. (a) The *Hind*III-*Xho*I subfragment of *Hind*III-P was 3' labeled at the *Hind*III site and used as a probe in S1 mapping experiments with the following (lanes): 3, probes alone (no RNA); 4, poly(A)⁺ RNA 6 h p.i.; 5, poly(A)⁺ RNA 24 h p.i.; 1 and 2, markers. (b) Location of the 1,500-base transcript with respect to the *Hind*III-Q and -P fragments.

FIG. 6. Mapping the 1,100-base transcript. (a) A 1,735-bp DNA probe, 5' labeled at the *Xho*I site, was made from the *Hind*III-Q fragment. (b) This probe was used for S1 mapping experiments with the following (lanes): 1 and 2, size-selected 1,500-base RNA; 3, RNA isolated from the region of the gel between the 1,500- and 1,100-base bands; 4, 1,100-base RNA; 5, no RNA; 6, untreated probe; 7 and 8, markers. (c) A 465-bp *Xho*I-*Hind*III subfragment of *Hind*III-Q was 3' labeled at the *Xho*I site. (d) S1 mapping experiments with this probe. Lanes: 3, mapping with poly(A)⁺ RNA 12 h p.i.; 1 and 2, markers. (e) Location of the 1,100-base transcript relative to the *Hind*III-Q and -P fragments.

FIG. 7. Location of four transcripts that map to the region of the 10K protein gene. The 1,100- and 1,500-base transcripts are in maximum abundance at 12 h p.i.; the 750- and 2,500-base transcripts are in maximum abundance at 24 h p.i.

Overlapping transcripts with different 5' ends are probably produced by RNA polymerase initiating at different sites. An alternative, but unlikely, explanation is that the 5' ends of these transcripts do not represent different transcription starts, but result from posttranscriptional end clipping.

Overlapping transcripts that share the same 5' end may be generated simply by utilization of different polyadenylation signals. A more difficult question is why the 3' ends of the γ transcripts never extend beyond the site in *Hind*III-P, 240 bases from the P-Q boundary, but some of the δ transcripts miss this same polyadenylation signal and end farther downstream in the P fragment.

Considering the experimental evidence regarding overlapping transcripts, the mechanism of their synthesis seems more comprehensible than their function. Lübbert and Doerfler (16) suggested that, since there are apparently no introns in the AcMNPV genes, overlapping transcripts could provide variety that might otherwise be provided by alternate splicing. Friesen et al. (11) suggested that promoter occlusion may be operating in AcMNPV, as follows. When different promoters are located on the same DNA strand, activation of the upstream promoter with the resulting transcription through the downstream promoter may interfere with initiation at the downstream promoter. Although promoter occlusion was untested in AcMNPV, the hypothesis was consistent with the location of overlapping transcripts that mapped to the *Hind*III-K fragment (11).

It is possible that promoter occlusion could also be operating in the region of the 10K protein gene. Synthesis of the earlier gene products, the 1,500- and 1,100-base transcripts, might prevent RNA polymerase initiation at the 10K gene promoter located downstream. At a later time, when transcription is no longer initiated at the upstream promoter, RNA polymerase would be able to initiate downstream at the promoter for the 10K protein gene. Our data do not rule this out, but neither do they allow us to conclude that synthesis of the earlier transcripts ends before synthesis of the later ones begins. Therefore, it is not clear that the promoter occlusion model applies to regulation of transcription in the region of the 10K protein gene.

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