Location, Transcription, and Sequence of a Baculovirus Gene Encoding a Small Arginine-Rich Polypeptide

MARIJO E. WILSON,[†] TERRY H. MAINPRIZE, PAUL D. FRIESEN, AND LOIS K. MILLER^{‡*}

Department of Bacteriology and Biochemistry, University of Idaho, Moscow, Idaho 83843

Received 15 August 1986/Accepted 17 November 1986

The nucleotide sequence of the cDNA of an abundant late 0.5-kilobase transcript of *Autographa californica* nuclear polyhedrosis virus revealed a small open reading frame encoding an arginine-rich 6.9-kilodalton protein. The predicted amino acid composition of the 6.9-kilodalton protein was essentially identical to that of the core protein of viral nucleocapsids. The precise location of the 5' and 3' ends of the transcript were confirmed by S1 nuclease and primer extension analyses. Multiple overlapping transcripts through this region include three early and three abundant late RNAs which are transcribed counterclockwise and one transient RNA which is transcribed clockwise with respect to the physical map of the virus.

Autographa californica nuclear polyhedrosis virus (AcNPV) serves as a vector for foreign gene expression (20), as a biological pesticide (22, 23), and as a model system for molecular biological analysis of baculoviruses (2). Current perceptions of gene organization within the 128-kilobase-pair (kbp) circular DNA genome of AcNPV are derived from in vitro translation of hybrid-selected viral mRNAs (1, 3, 21, 27), DNA sequencing of selected regions of the DNA genome (9, 13), and transcriptional studies (5, 15, 16, 24, 28). The DNA sequences of two AcNPV genes, one encoding the polyhedrin gene and the other encoding a late nonstructural 10,000-molecular-weight (10K) protein, reveal uninterrupted coding regions flanked by A+T-rich sequences (9, 13). Multiple overlapping unspliced RNAs with common 3' or 5' termini are frequent motifs of AcNPV transcription (5, 16, 24).

In this report we provide the location, sequence, and transcriptional patterns of a gene which is abundantly transcribed late in infection. The sequence reveals an uninterrupted open reading frame (ORF) encoding an arginine-rich 6.9-K polypeptide. The amino acid composition of the predicted 6.9K protein is essentially identical to the empirically determined amino acid composition of the arginine-rich core protein constituting over half of the total nucleocapsid protein (12, 30). This protein is closely associated with the viral DNA in the core (30) and appears to be phosphorylated during the uncoating process within the nucleus (34).

MATERIALS AND METHODS

Recombinant plasmids and DNA sequencing strategy. Plasmid pHD2 contains an AcNPV cDNA insert of approximately 0.5 kb in a pUC9 vector (16a). The insert hybridizes to the *Hind*III-*H*/*Eco*RI-*D* region spanning 66.2 to 66.8 map units on the AcNPV physical map. The HD2 insert was subcloned into M13 in both orientations by using the *Pst*I and *Bam*HI sites on the pUC9 vector and an *Xma*I site within the insert. Sequencing across the *Xma*I site was accomplished by using a subclone from the *Bam*HI site of the pUC9 vector proximal to the poly(A) tail of the HD2

insert to a *Kpn*I site in the insert. Exonuclease III (8) was used to eliminate the poly(A) tail so that sequence information could be obtained on the opposite strand reading toward the *Xma*I site. To sequence the genomic DNA corresponding to the HD2 cDNA, the entire *Hind*III-*H* region of AcNPV was cloned into the *Hind*III site of pACYC177 to yield pMW-H28. The two viral *Hind*III-*Xma*I fragments of pMW-H28 were subcloned into M13 for sequencing. The DNAs were sequenced by the dideoxynucleotide chain termination method with a sequencing kit purchased from Bethesda Research Laboratories (Gaithersburg, Md.) with [³⁵S]dATP (New England Nuclear Corp., Boston, Mass.).

RNA isolation and Northern blot hybridization analysis. RNA was isolated (16a) at various times postinfection (p.i.) from monolayers of *Spodoptera frugiperda* IPLB-SF21 cells infected at a multiplicity of 20 PFU/cell with AcNPV-L1 (14). Starting 1 h prior to infection, cycloheximide-treated cells were incubated in medium containing cycloheximide (100 µg/ml); this concentration of drug was maintained during inoculation and throughout the infection. Oligo(dT)-cellulose chromatography was used to select polyadenylated RNA (10). For Northern blot analysis, polyadenylated RNA (2 to 10 µg) from 10⁷ cells was denatured by glyoxalation, electrophoretically separated in agarose gels (18), and transferred to nitrocellulose filters. Hybridization (29) was performed with recombinant plasmids containing HD cDNA inserts radiolabeled by nick translation (17) or with single-stranded recombinant M13 phage DNA radiolabeled by primer extension (11) as described previously (19).

S1 nuclease and primer extension analyses of the HD transcripts. The 5' and 3' ends of the 0.5-kb RNA transcript were mapped by S1 nuclease analysis (33). The 5'-end-labeled probe was generated by cleaving pMW-H28 with *Xma*I; this generated a 2,150-base-pair (bp) fragment and a 5,620-bp fragment because there is an *Xma*I site within the pACYC177 vector. (Note that the 2,150-bp fragment generated by *Xma*I digestion is coincidentally the same size as an *Xma*I-*Hind*III fragment shown in Fig. 4). The 2,150-bp fragment was purified electrophoretically. The 5' end was dephosphorylated with calf intestinal phosphatase and radiolabeled with T4 polynucleotide kinase and [^γ-³²P]ATP (3,000 Ci/mmol) (17). The DNA was cleaved with *Hind*III, and a 1,950-bp fragment was isolated that was radiolabeled exclusively on one strand at the *Xma*I site (see Fig. 4). To prepare the 3'-specific S1 probe, the 5,620-bp *Xma*I fragment of

* Corresponding author.

[†] Present address: Department of Entomology, University of Kentucky, Lexington, KY 40546.

[‡] Present address: Departments of Genetics and Entomology, University of Georgia, Athens, GA 30602.

FIG. 1. Northern blot hybridization analysis of RNA homologous to the cDNA-containing plasmid pHD2. Polyadenylated RNA isolated at 2, 6, 12, and 24 h p.i. was glyoxalated and electrophoretically separated on a 1.7% agarose gel. The RNA was transferred to nitrocellulose, and the blot was hybridized to radiolabeled pHD2 DNA. An autoradiogram of the blot is shown (top). Glyoxalated molecular size standards of restriction endonuclease-digested plasmid pBR322 are indicated at the left (in kilobases), and the sizes of major RNAs are indicated on the right (in kilobases). The 500-bp HD2 cDNA insert (open box) was mapped to the *KpnI* and *XmaI* sites within the *HindIII*-H fragment of AcNPV as indicated.

pMW-H28 was labeled with T4 polymerase and [α - 32 P]dCTP (3,000 Ci/mmol) (17). The fragment was then cleaved with *HindIII* to yield a 2,150-bp fragment (Fig. 4). DNA-RNA hybridization mixtures consisting of 2 μ g of polyadenylated RNA and approximately 100 ng of labeled DNA were conducted at 48°C for 6 h (5, 33). The S1 hybrids were analyzed by electrophoresis on 6% polyacrylamide wedge sequencing gels. *MspI* digests of pUC19 plasmid DNA (31) were 3'-end labeled and used as size standards. A sequencing ladder of the HD region provided precise information on location.

For primer extension analysis, the 57-bp primer was derived from the 1,950-bp S1 probe by cutting with *KpnI* and purifying the small fragment (Fig. 4). The primer was annealed to 2 μ g of polyadenylated RNA under hybridization conditions identical to those for S1 analysis. Following primer extension (5), the products were resolved on a 6% polyacrylamide sequencing gel.

RESULTS

In a previous report (16a) we described a series of AcNPV cDNAs isolated at 2 h and 10 h p.i. The predominant viral cDNAs in the 10-h library were those hybridizing to the *HindIII*-H/*EcoRI*-D (HD) region of the AcNPV genome (62.7 to 66.8 map units). A cDNA clone, pHD2, containing a 0.5-kb insertion was selected for further study.

Northern blot analysis of transcripts homologous to the HD cDNA. Three abundant RNAs of 0.5, 2.0, and 3.15 kb homologous to pHD2 were detected in AcNPV-infected cells (Fig. 1). All transcripts appeared by 12 h p.i. and were

maximally expressed at 24 h p.i. Unlike the polyhedrin and p10 genes, the 0.5-kb RNA was abundant at 12 h as well as 24 h. A strand-specific probe (Fig. 2, bottom) was used to detect RNA transcribed counterclockwise with respect to the circular genome and revealed four transcripts at 8 h p.i. (Fig. 2a). The 1.35-, 1.6-, and 1.75-kb transcripts were expressed in cycloheximide-treated cells (prolonged exposure), while the 0.5-kb transcript was only expressed in untreated cells. At 24 h p.i., two additional transcripts, the 2.0- and 3.15-kb transcripts, were observed. The strand-specific probe used to detect RNA transcribed clockwise with respect to the circular genome hybridized to a 2.0-kb transcript at 8 h p.i. This transcript was not expressed in the presence of cycloheximide and was no longer present at 24 h p.i.

DNA sequences of the HD2 insert and the equivalent genomic region. The nucleotide sequence of the HD2 cDNA insert (Fig. 3) revealed a 381-bp virus-encoded sequence with a poly(A) tail of approximately 120 nucleotides. The equivalent genomic HD sequence was identical to the cDNA sequence [minus the poly(A) tail].

Location of the 5' and 3' ends of the HD2 mRNA by S1 nuclease and primer extension analysis. The sequence of the HD2 cDNA and strand-specific Northern blot analysis indicated that transcription of the 0.5-kb RNA proceeded counterclockwise on the circular genome. Polyadenylated RNA isolated from AcNPV-infected cells at 24 h p.i. was hybridized to the 1,950-bp *XmaI*-*HindIII* fragment labeled at the 5' end at the *XmaI* site (Fig. 4a). An S1 nuclease-resistant fragment of 131 nucleotides was generated (Fig. 4b and c). This corresponded to the 5' end of the HD2 cDNA insert. Primer extension of a 57-bp *XmaI*-*KpnI* primer, labeled at the *XmaI* site at the 5' end, resulted in a 131-bp extension

FIG. 2. Northern blot hybridization analysis of RNA homologous to strand-specific pHD2 probes. (Top) Polyadenylated RNA isolated at 8 h in the presence of cycloheximide (8+CX) or at 8 and 24 h p.i. in the absence of cycloheximide were treated as described in the legend to Fig. 1 and hybridized with strand-specific probes of the HD2 cDNA insert. (a) 304-base 5' to 3' clockwise probe; (b) 253-base 5' to 3' counterclockwise probe. A longer exposure of the 8+CX lane (a) is included on the far left. Molecular size standards are indicated on the far right and the sizes of the RNAs are indicated in the center between lanes (in kilobases). (Bottom) Locations of the 304-base clockwise (a) and 254-base counterclockwise (b) single-strand-specific probes (striped arrows).

product as well as a 130-bp product. The 5' end determined by both primer extension (131-bp product) and S1 nuclease analysis (Fig. 4c) corresponds exactly with the 5' end of the HD2 cDNA. Transcription initiating from this site is sensitive to the effects of cycloheximide, although low levels of transcription can be detected even in the presence of the drug.

The 3' end of the transcript was determined by S1 nuclease analysis with a 2,150-bp *XmaI*-*HindIII* fragment labeled exclusively at the *XmaI* site on the 3' end. The HD2 cDNA sequence predicted that the 3' end of the transcript would lie approximately 250 bp downstream from the *XmaI* site, and this was substantiated in the S1 protection experiment in which an S1-resistant fragment of 253 bases was observed (Fig. 4a and b). Prolonged exposure of the autoradiogram (Fig. 4b) showed protected DNA larger than 253 bases in the 24-h RNA lanes but not the lanes from 8-h RNA produced in the presence of cycloheximide (data not shown). This finding indicates that RNAs extending downstream from the 3' end of the HD2 transcript were present at 24 h p.i. in the absence of cycloheximide but not at 8 h in the presence of cycloheximide, suggesting that the 2.0- and 3.15-kb late counterclockwise transcripts were 5'-coterminal with the 0.5-kb HD2

transcript but had extended 3' ends. The positions of the 3' ends of these other late transcripts were not determined. It may also be noted that the intensity of the 3' S1 nuclease signal for RNA produced in the presence of cycloheximide was the same as the signal produced by RNA isolated at 24 h in the absence of cycloheximide. This finding suggests that the cycloheximide-resistant counterclockwise transcripts utilize the same 3'-end signal as the late transcripts. Further work will be necessary to determine the organization of these early RNAs.

DISCUSSION

The largest methionine-initiated ORF within the abundantly transcribed region represented by the HD2 cDNA clone is 55 codons. Such a short coding sequence could easily be overlooked, but three features of the sequence suggest that this ORF is translated: (i) the ATG of this ORF is the first ATG from the 5' end of the polyadenylated RNA, (ii) the ATG is preceded by the sequence AAC, and (iii) codon usage bias of the ORF indicates that it is in all probability translated (4). The arginine codon usage bias of the ORF is identical to that of the polyhedrin and p10 genes (26a).

The predicted amino acid sequence of the ORF reveals that the peptide is rich in arginine (40%). Tween et al. (30) observed an extremely basic, arginine-rich polypeptide associated with the DNA in the core of baculovirus particles. Kelly et al. (12) determined the amino acid composition of the basic protein of *Trichoplusia ni* NPV (TnNPV), a genotypic variant of AcNPV. Table 1 compares the predicted amino acid composition of the ORF with that of the basic core protein of TnNPV. The proteins appear to be identical from the close correspondence of their very unusual base compositions.

Like other basic core proteins, the HD 6.9K protein is arginine-rich, lysine-poor, and rich in helix-destabilizing residues. Although the predicted molecular weight is lower than that estimated by Tween et al. (30), highly basic proteins often exhibit aberrant migration on sodium dodecyl sulfate-polyacrylamide gels. Kelly et al. proposed that the protein interacts with DNA by ionic interactions of the arginine residues with the negatively charged phosphosugar backbone of the DNA helix and by intercalation of the tyrosine residues among the stacked base pairs (12). Although both types of residues are found throughout the polypeptide chain, the arginine residues are partially clustered at the amino terminus, whereas the tyrosines are partially clustered at the carboxyl terminus.

The gene for the 6.9K protein is transcribed into at least three different RNAs. The major 0.5-kb transcript of the 6.9K protein gene is highly abundant at late times in infection and is one of the three most abundant viral transcripts found between 24 h and 30 h p.i. (26). It is transcribed counterclockwise with respect to the circular genome. Two larger RNAs of 2.0 and 3.15 Kb appear to be transcribed in the same direction from the same promoter. The S1 and primer extension data indicate a single 5' end for all counterclockwise late transcripts; S1 data also suggest extended 3' ends at late times p.i. Overlapping 5'-coterminal transcripts appear to be characteristic of late, highly transcribed viral RNAs (5, 16, 28) and may be due to a scarcity of transcriptional termination factors late in infection. All three of these transcripts are sensitive to cycloheximide, indicating that their transcription is dependent on one or more viral proteins.

FIG. 3. Nucleotide sequence of the HD2 region of AcNPV. The DNA sequence of the HD2 cDNA and upstream regions derived from sequencing the genomic region are presented. The 5' end of the major 0.5-kb transcript is indicated at +1, and the 3' end is within a base or two of the last nucleotide. The polyadenylation signal is underlined. A series of direct repeats are shown by arrows and bars above the repeated sequences. The largest methionine-initiated ORF is translated below the sequence (+41 to +205). The restriction sites for *XmaI* and *KpnI* are shown.

FIG. 4. Mapping the 5' and 3' ends of the 0.5-kb HD transcript by S1 nuclease and primer extension analysis. (a) Nature and locations of the hybridization probes and the primer used for mapping. The position of the radiolabel is indicated by asterisks. (b) Polyadenylated RNA (2 μ g) was isolated from cycloheximide-treated cells at 8 h p.i. (lanes 2, 3, 7, and 8) and from untreated cells at 24 h p.i. (lanes 4, 5, 9, and 10) and hybridized to either the 5'-end-labeled 1,950-bp probe (lanes 1 to 5) or to the 3'-end-labeled 2,150-bp probe (lanes 6 to 10). The DNA-RNA hybrids were treated with either 20 (lanes 2, 4, 7, and 9) or 200 (lanes 3, 5, 8, and 10) U of S1 nuclease per ml. The S1-resistant fragments were denatured and separated on a sequencing gel. An autoradiogram (12-h exposure) is shown. Molecular sizes markers are indicated on the right (in nucleotides). (c) Polyadenylated RNA (2 μ g) isolated from cells at 8 h p.i. in the presence of cycloheximide (lane 1) or 8 and 24 h p.i. in the absence of cycloheximide (lanes 2 and 3, respectively), and 2 μ g of tRNA (lane 4) were annealed to the 57-base *XmaI-KpnI* primer and extended with reverse transcriptase. An autoradiogram (24-h exposure) of the sequencing gel used to separate the DNAs by size is shown. S1-resistant fragments generated from the hybridization of RNAs as described for panel b are shown on the right. Lanes 1 and 2, 8-h RNA in the presence of cycloheximide; lanes 3 and 4, 24-h RNA. In the center is a sequencing ladder generated by treating the 57-bp primer with exonuclease III, annealing it to the M13 recombinant plasmid containing the genomic sequences, and extending it with Klenow fragment polymerase in the presence of four deoxyribonucleotides (A, C, G, and T).

Overlapping these three late-abundance RNAs are three polyadenylated early RNAs that do not require active synthesis of viral proteins for their transcription, based on their insensitivity to cycloheximide. The termini of these RNAs were not rigorously mapped in this study, but there is evidence that an ORF lies just clockwise (upstream) of the major 0.5-kb transcript (S. Thiem and L. K. Miller, unpublished results). A very minor, transient, cycloheximide-

sensitive polyadenylated RNA is transcribed from the opposite strand through the 6.9K protein gene region.

A comparison of the sequences near the 5' end of the 0.5-kb RNA with other sequenced promoter regions reveals several interesting features. The sequence ATAAG is found at the 5' end. This sequence is highly conserved at the 5' ends of both the polyhedrin and p10 genes of different baculoviruses (26a). However, other conserved sequences

TABLE 1. Amino acid composition of the predicted 6.9K protein and the basic core protein of TnNPV

Amino acid	Content (mol%)	
	6.9K protein	TnNPV core protein
Ser	18.1	16.6
Gly	9.0	12.9
Arg	40.0	31.0
Tyr	12.6	10.4
Glu	0.0	1.9
Cys	0.0	1.9
His	0.0	— ^a
Asp	0.0	2.1
Thr	12.6	11.2
Met	2.0 ^b	1.0
Pro	4.0	4.3
Ala	0.0	2.1
Val	2.0	2.8
Lys	0.0	0.8
Leu	0.0	0.8
Cysa	0.0	—
Phe	0.0	—
Ile	0.0	—

^a —, Negligible amounts observed; data from Kelly et al. (12).

^b Actual value depends on whether the methionine residue is retained in the mature protein.

surrounding this central sequence are not found in the 6.9K protein gene and may account in part for the differential temporal regulation of these genes. Transcription of the 0.5-kb transcript begins at the second A in the ATAAG sequence; in our hands, this is also the start site for the major AcNPV polyhedrin RNAs, as determined by primer extension analysis (M. E. Wilson and L. K. Miller, unpublished observations). Comparison with the promoter region of an abundant 39K protein (6) shows that the sequence AGCGTAAAAA (–18 from the 5' end) is conserved (AGCGTATAAAA at –23 from the 5' end of the late RNA of the 39K protein gene). The sequence TAAAAAATAT, found 14 bp upstream of the HD RNA start site, is found within the late leader of the 39K protein gene.

At least three sequence elements are directly reiterated in slightly different spatial arrangements just upstream of the 6.9K protein coding sequence (Fig. 3). There are 175 untranslated bases at the 3' end of the 0.5-kb transcript of the 6.9K protein gene. A typical polyadenylation signal, AAUAAA, precedes the polyadenylation site by 12 nucleotides (Fig. 3). The coding sequence of the 6.9K protein gene is flanked by extremely A+T-rich sequences. This arrangement is similar to that observed for both the polyhedrin and p10 genes and may be characteristic of baculovirus genes in general.

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