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Identification and Sequence Analysis of a Gene Encoding gp67, an Abundant Envelope Glycoprotein of the Baculovirus *Autographa californica* Nuclear Polyhedrosis Virus

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Monoclonal antibodies with specificity for the abundant envelope surface glycoprotein (gp67) of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) were used to screen a lambda gt11 expression library of Ac*M*NPV DNA fragments. The gp67 gene was mapped to the left end of the *Eco*RI H fragment in a right-to-left orientation on the consensus map of Ac*M*NPV. A 2.1-kilobase transcript which hybridized to the region was first detected in cell extracts at 2 h postinfection; it peaked in abundance at 18 h postinfection and thereafter was present at lower levels. The nucleotide sequence of the region was determined, and a 1,590-nucleotide open reading frame flanked by an AT-rich sequence was identified that could encode a polypeptide with 529 amino acid residues (molecular mass of 60,167 daltons). Computer analysis indicated that the peptide possesses two hydrophobic regions near the N and C termini as well as six potential N-linked glycosylation sites. We suggest that following cleavage of a signal peptide, the polypeptide undergoes further processing and becomes anchored at its C terminus in the virus envelope. The final seven amino acid residues at the C terminus contain basic amino acids and may have a role in virion assembly.

Two virion phenotypes are produced during replication in insects and in cell culture of the baculovirus *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV). Occluded virus (OV) particles are enveloped nucleocapsids embedded within occlusion bodies which form in infected cell nuclei. This virus phenotype is necessary for transmission of infection between insects (6, 11). The budded virus (BV) phenotype arises when nucleocapsids bud through the plasma membrane. In insects, BV is the vehicle for systemic spread of the infection. Infectivity of the two forms differs in vivo. BV is more infectious than OV if injected into the hemocoel, and OV is more infectious than BV when injected into the gut (14, 42). BV is about 1,800 times more infectious in cultured TN-368 cells than is OV (43). The presence of an abundant envelope glycoprotein in BV (here designated gp67) was considered by Keddie and Volkman (14) to contribute to the observed differences in infectivity.

gp67 is an acidic protein (pI, 3.15) (39) with reported molecular weights ranging from 64,000 to 70,000 (38). It is glycosylated (10, 33, 41), contains asparagine-linked carbohydrate moieties (33, 41), and is phosphorylated (22, 39). Immunolabeling of infected cells provided direct evidence that gp67 is a component of the virus peplomers (41). It is the target of a neutralizing monoclonal antibody, AcV1 (12, 41) and is considered to be essential for the entry of BV by adsorptive endocytosis by functioning at the penetration step and possibly playing a role in low pH-dependent fusion (40).

In this study we used monoclonal antibodies which specifically bound gp67 to select clones from a lambda gt11 expression library of Ac*M*NPV DNA. Recombinant bacteriophage was used to probe the genomic DNA to locate the gene and to identify gp67 mRNAs. Appropriate fragments were sequenced, and the deduced amino acid sequence is given.

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MATERIALS AND METHODS

Virus and cell culture. A plaque-purified isolate HR3 (2) of Ac*M*NPV was propagated in *Spodoptera frugiperda* IPLB-SF-21 cells (36). BV was concentrated from HR3-infected culture supernatants by pelleting through a 20% sucrose cushion. The murine myeloma line Sp2/0-Ag14 (30) and hybridoma cells were propagated in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 1 mM glutamine, 0.05 mM 2-mercaptoethanol, and 10 μg of gentamycin per ml.

Production of monoclonal antibodies. BALB/c mice were given a primary intraperitoneal injection of 25 μg of BV in Freund complete adjuvant, followed by a second intraperitoneal injection of 25 μg of BV in Freund incomplete adjuvant 2 weeks later. After a rest period of at least 2 weeks, the mice were injected intraperitoneally with 100 μg of virus in phosphate-buffered saline on three consecutive days, and their spleens were removed on day 4.

Fusion of mouse spleen cells and Sp2/0-Ag14 myeloma cells was performed as previously described (7). Hybridoma culture supernatants were screened for the production of virus-specific antibody by an enzyme-linked immunosorbent assay with BV as antigen. Hybridomas secreting BV-specific antibodies were cloned twice by limiting dilution. Culture supernatants from cloned hybridomas were used to determine the polypeptide specificity of the monoclonal antibodies.

Immunoblot analysis. BV polypeptides were resolved by electrophoresis in 12% sodium dodecyl sulfate (SDS)-polyacrylamide slab gels (18) and were electrophoretically transferred to nitrocellulose membranes (35). Membranes were blocked overnight with 3% bovine serum albumin and washed with 0.05% Tween 20 in phosphate-buffered saline. The nitrocellulose strips were incubated for several hours with undiluted hybridoma supernatant. After being washed, the strips were incubated with a 1:300 dilution of biotinylated goat anti-mouse immunoglobulin (Amersham Corp., Arling-

TABLE 1. Characterization of monoclonal antibodies against gp67

Antibody	Isotype	Molecular mass (10^3) of antigen(s) recognized by[a]:	
		Immunoblot	Immunoprecipitation
AcN1	IgG2b	67	67
AcN2	IgG2a	67	67
AcN3	IgG2b	67 (42)	67
AcN4	IgM	67 (42)[b]	67
AcN5	IgG3	67 (77,42)[b]	67
AcN6	IgG2b	67	—[c]
AcN7	IgG2a	67	—
AcN8	IgG3	67	—
AcN9	IgG2b	67	—
AcN10	IgG2a	67 (42)	—
AcN11	IgG1	—	67
AcN12	IgG1	—	67
AcN13	IgG2a	—	67
AcN14	IgG2a	—	67 (52)
AcN15	IgG1	—	67 (52)
AcN16	IgG2a	—	67 (52)

[a] Values represent molecular mass in kilodaltons, with weak reactivities in brackets.
[b] Monoclonal antibodies bound to an antigen that ran with the tracking dye.
[c] —, No reaction.

ton Heights, Ill.). Bands were visualized by incubating the strips with streptavidin-biotinylated enzyme preformed complex and then with a solution of 0.05% 4-chloro-1-naphthol.

Immunoprecipitation. Pellets of BV labeled with [^{35}S] methionine were sonicated in 200 μl of solubilization buffer (0.01 M Tris hydrochloride [pH 7.6], 0.15 M NaCl, 0.001 EDTA, 0.002 M phenylmethylsulfonyl fluoride, 0.02% sodium azide) containing 1% Triton X-100. Precipitation was performed by using undiluted hybridoma supernatants and Protein A-Sepharose CL-4B (39).

Construction and screening of lambda gt11 expression library. Ac*M*NPV HR3 DNA was isolated from BV by SDS-proteinase K digestion and phenol extraction (34) and was sheared by sonication for 30 s. After methylation with *Eco*RI methylase (Promega Biotec, Madison, Wis.), the DNA fragments were made blunt ended with T4 DNA polymerase and were ligated to phosphorylated *Eco*RI linkers (Pharmacia, Inc., Piscataway, N.J.). After treatment with *Eco*RI, excess linkers were removed by chromatography on Sephadex G-50 (Pharmacia), and the HR3 DNA fragments were ligated to *Eco*RI-cleaved, dephosphorylated lambda gt11 arms (Promega Biotec). Ligated recombinant DNA was packaged in vitro (Amersham) and used to infect *Escherichia coli* Y1090 cells.

The library was screened with monoclonal antibodies essentially as described previously (13). Immunoreactive phage were picked and purified by four subsequent steps of cloning and screening. Purified phage DNA was prepared from plate lysates by using LambdaSorb (Promega Biotec).

Southern blot analysis. Restriction endonuclease fragments of HR3 DNA were separated on 0.8% agarose gels and transferred to nitrocellulose sheets by using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (31). Hybridization probes were prepared from lambda gt11 recombinant phage expressing epitopes recognized by the monoclonal antibodies. Phage DNA was biotinylated by nick translation with biotin-7-dATP as described for the BluGENE system (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Hybridization, washes, and detection were performed as described by the manufacturer.

RNA extraction and Northern (RNA) blot analysis. Total cellular RNA was isolated at various intervals postinfection (p.i.) from IPLB-SF-21 cells infected with HR3 at a multiplicity of 10 PFU per cell by using guanidine isothiocyanate (5). For Northern blot analysis, 30 μg of total cellular RNA was denatured by glyoxylation, separated in 1.2% agarose gels (23), and transferred to nitrocellulose sheets by using 20× SSC. Probes were the same phage strains used for Southern blot analysis except that they were radiolabeled with [α-^{32}P]dCTP by nick translation. Hybridization was done overnight at 42°C by using 2×10^7 cpm of the probe in the presence of 50% formamide, 5% dextran sulfate, 5× SSC, Denhardt solution, 0.05 M sodium phosphate (pH 6.5), and 100 μg of denatured herring sperm DNA per ml. After hybridization, filters were washed three times in 2× SSC–0.1% SDS for 5 min each at room temperature and three times in 0.1× SSC–0.1% SDS for 15 min at 55°C. Filters were dried and exposed to Kodak XAR5 film.

DNA sequence analysis. The *Eco*RI H fragments were subcloned from pBR322 containing the fragment (3) into M13mp18 and M13mp19. A series of overlapping clones were then generated by using the CYCLONE system (International Biotechnologies, Inc., New Haven, Conn.) (4). Sequencing was performed by using dideoxynucleotide chain terminator sequencing (29). In some cases, synthetic primers generated by the Oligonucleotide Synthesis Laboratory, Department of Biochemistry, Queen's University, were used. Computer analysis was done by using the programs of Staden (32) and Pearson and Lipman (25).

RESULTS

Characterization of monoclonal antibodies to gp67. Hybridoma cell lines were made by using spleen cells from mice immunized with Ac*M*NPV BV. Sixteen lines produced monoclonal antibodies that bound an abundant 67,000-molecular-weight BV structural polypeptide (67K polypeptide) as measured by immunoblotting or radioimmunoprecipita-

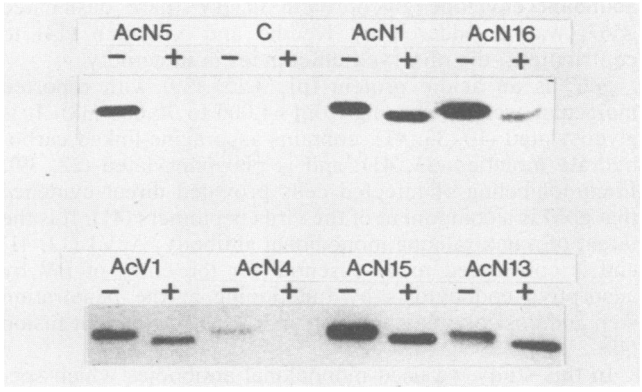

FIG. 1. Immunoprecipitation of Ac*M*NPV BV proteins by using specific monoclonal antibodies against gp67. [^{35}S]methionine-labeled BV proteins were solubilized and either left untreated (−) or digested with endoglycosidase F (+) before immunoprecipitation by monoclonal antibodies. Proteins were separated by SDS-polyacrylamide gel electrophoresis with 12% gels. AcV1 is a neutralizing monoclonal antibody that binds gp67 (12). In the two lanes under C (control), an irrelevant monoclonal antibody was used for immunoprecipitation.

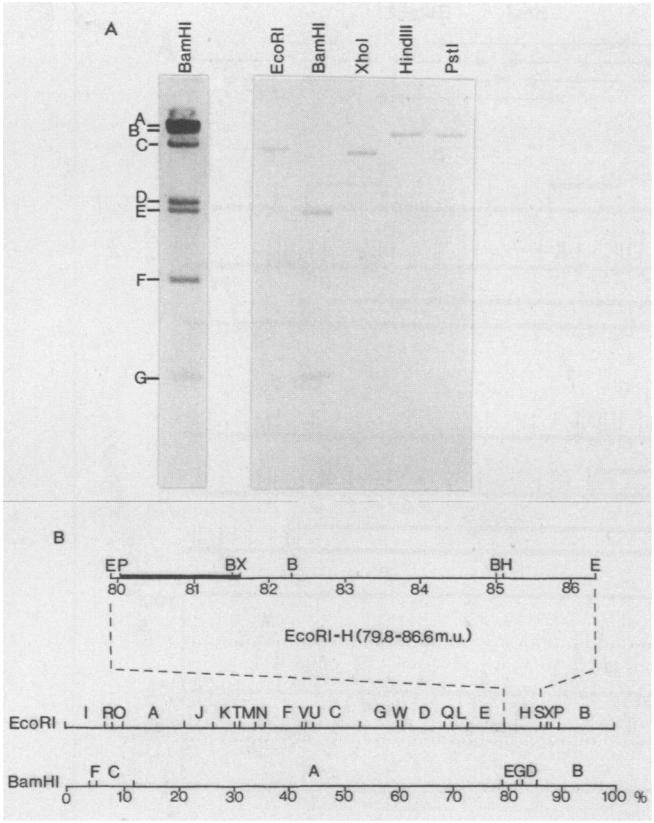

FIG. 2. Mapping of the gene encoding gp67 from recombinant phage. (A) Southern blot of Ac*M*NPV DNA digested with restriction enzymes and hybridized with nick-translated total virus DNA (extreme left lane) or nick-translated DNA from recombinant phage 67.5. *Bam*HI fragments are shown. (B) Restriction endonuclease maps for *Eco*RI and *Bam*HI. Detail shows *Eco*RI-H, with the heavy line indicating the approximate map position containing the gp67 sequences expressed by lambda gt11 recombinants and the following restriction sites: E, *Eco*RI; P, *Pst*I; B, *Bam*HI; X, *Xho*I; H, *Hin*dIII.

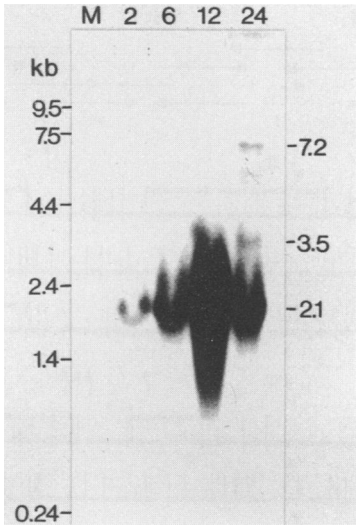

FIG. 3. Northern blot analysis of RNA homologous to immunoreactive lambda gt11 clones. Total cellular RNA isolated at 2, 6, 12, and 24 h p.i. was glyoxylated and separated by electrophoresis on 1.2% agarose gels. The RNA was transferred to nitrocellulose and hybridized to radiolabeled phage 67.1 DNA. Lane M, Mock-infected cells. Glyoxylated RNA size standards are indicated on the left (in kilobases). Calculated sizes of the RNAs are indicated on the right (in kilobases).

tion, or both (Table 1). The protein is considered to be the major glycoprotein on the surface of the BV phenotype (38). The molecular weight of gp67 decreased from 67,000 to 60,000 upon digestion of solubilized BV with endoglycosidase F (Fig. 1). A previously characterized neutralizing monoclonal antibody, AcV1 (12), also immunoprecipitated the same endoglycosidase F-sensitive 67K glycoprotein (Fig. 1).

Map position of the gene encoding gp67. Hybridoma supernatants containing two monoclonal antibodies, AcN8 and AcN9, which exhibited strong reactivity with gp67 in immunoblots were pooled and used to screen a lambda gt11 Ac*M*NPV DNA expression library. Approximately 5×10^5 plaques were screened, and seven recombinant phage were isolated which expressed epitopes recognized by the AcN8-AcN9 monoclonal antibody pool. These plaques were picked, cloned, and screened four times. Recombinant phage 67.1, 67.5, and 67.6 were selected for use in further studies.

To determine the map location of the gene encoding gp67, purified DNA from the three recombinant phage was nick translated with biotinylated nucleotides and was used to probe blots of Ac*M*NPV DNA digested with several restriction enzymes. Initial experiments indicated that the recombinants hybridized to regions of the Ac*M*NPV genome contained within the *Eco*RI H fragment. Subsequent investigation revealed that all three probes hybridized to a series of overlapping restriction fragments including *Eco*RI-H, *Hin*dIII-A, *Pst*I-B, and *Xho*I-G (Fig. 2). Two probes, 67.1 and 67.5, hybridized to adjacent *Bam*HI fragments E and G (Fig. 2), while 67.6 recognized *Bam*HI-E only (data not shown). This pattern indicated that the epitopes recognized by the monoclonal antibodies were encoded within the left half of *Eco*RI-H between the *Pst*I site at 80.1 map units (mu) and the *Xho*I site at 81.6 mu for phage 67.1 and 67.5 and between 80.1 mu and the *Bam*HI site at 81.5 mu for phage 67.6.

Characterization of transcripts from the *Eco*RI H region. To determine if the region of the genome thought to encode p67 was actively transcribed, Northern blot analysis was done to identify transcripts that hybridized with the recombinant probes. Total cellular RNA was separated by electrophoresis, blotted, and probed with recombinant phage DNA labeled with ^{32}P. A transcript 2.1 kilobases in size was detected in infected cells as early as 2 h p.i. (Fig. 3). It increased in abundance until 12 h p.i. but had decreased in amount by 24 h p.i. However, by 24 h p.i., two larger transcripts of 3.5 and 7.2 kilobases were also detected in cells (Fig. 3).

Nucleotide sequence, analysis of p60 ORF, and putative polypeptide structure. For sequencing, the *Eco*RI H fragments were subcloned into phage M13, and a series of overlapping clones was generated. In some instances, synthetic primers were used to sequence selected portions of the fragment (Fig. 4A). A region at the left end of the Ac*M*NPV *Eco*RI H fragment (79.8 to 81.9 mu) 2,684 nucleotides in length was sequenced (Fig. 5). Computer analysis revealed 10 open reading frames (ORFs) greater than 20 amino acids in length (Fig. 4B). The largest is 1,590 base pairs and encodes 529 amino acid residues (p60 in Fig. 5). It runs from right to left by using the conventional orientation for the

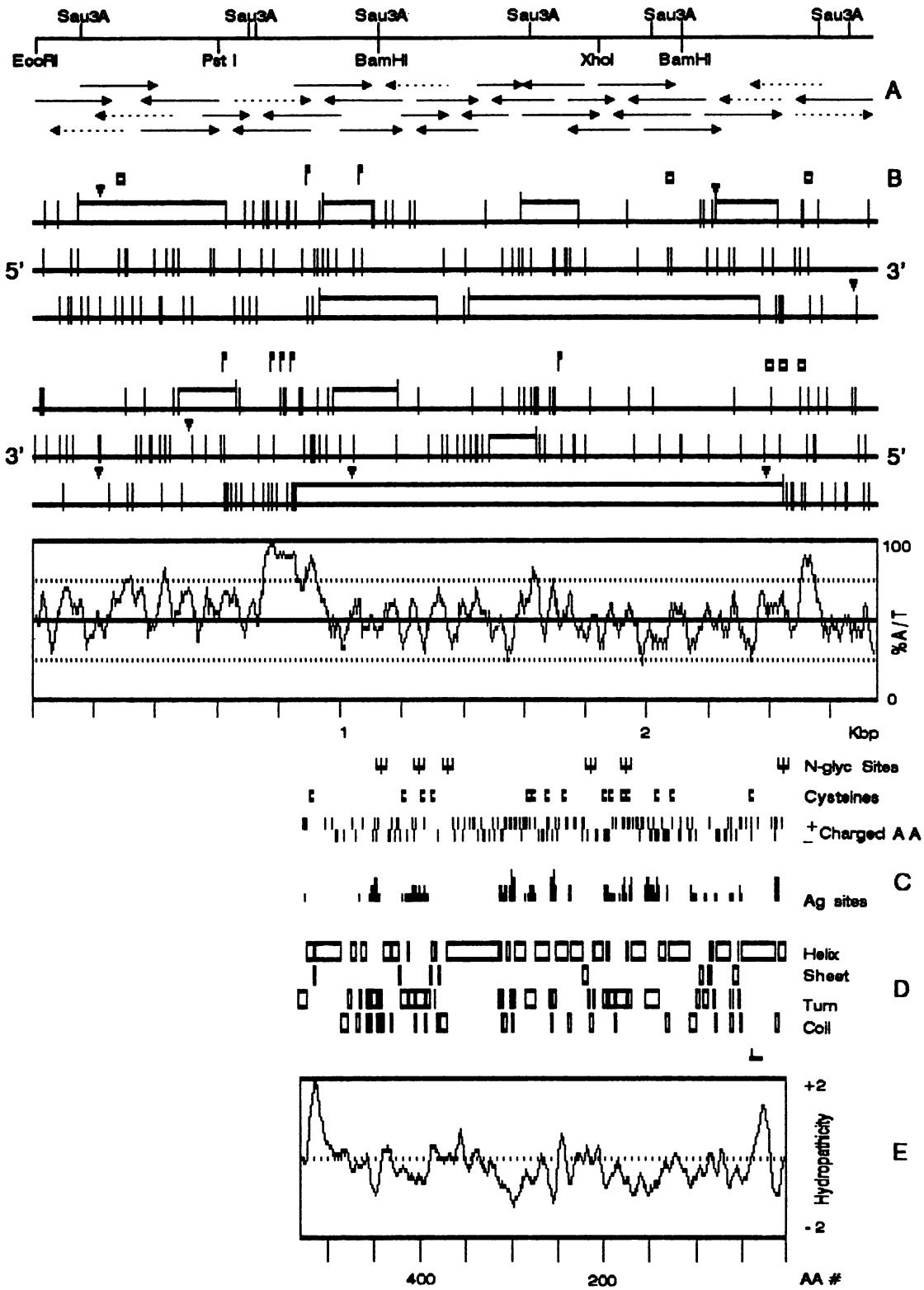

FIG. 4. Sequence analysis of the left end of the *Eco*RI H (79.8- to 81.9-mu) fragment of the Ac*M*NPV genome. (A) Sequencing strategy. Segments designated with solid arrows were sequenced with commercial M13 primers. Segments with dashed arrows were sequenced by using synthetic oligonucleotide primers. (B) ORF analysis showing all ORFs with a coding potential greater than 20 amino acids. Polyadenylation signals ( ), Kozak consensus initiation codons (▼), and potential baculovirus late mRNA cap sites ( ) are shown. The percent A+T for the sequence was determined as an average by using a 50-nucleotide moving window. The 5′ to 3′ (+) and 3′ to 5′ (−) directions are shown. (C) Putative N-glycosylation sites ( ), cysteines ( ), and charged amino acid and potential antigenic sites are shown. (D) Secondary structure prediction for p60 is based on the method of Garnier et al. (9). (E) Hydropathicity profile of the predicted p60 amino acid sequence was performed by using the methods of Kyte and Doolittle (17). A predicted signal cleavage site ( ) is marked above this plot (45).

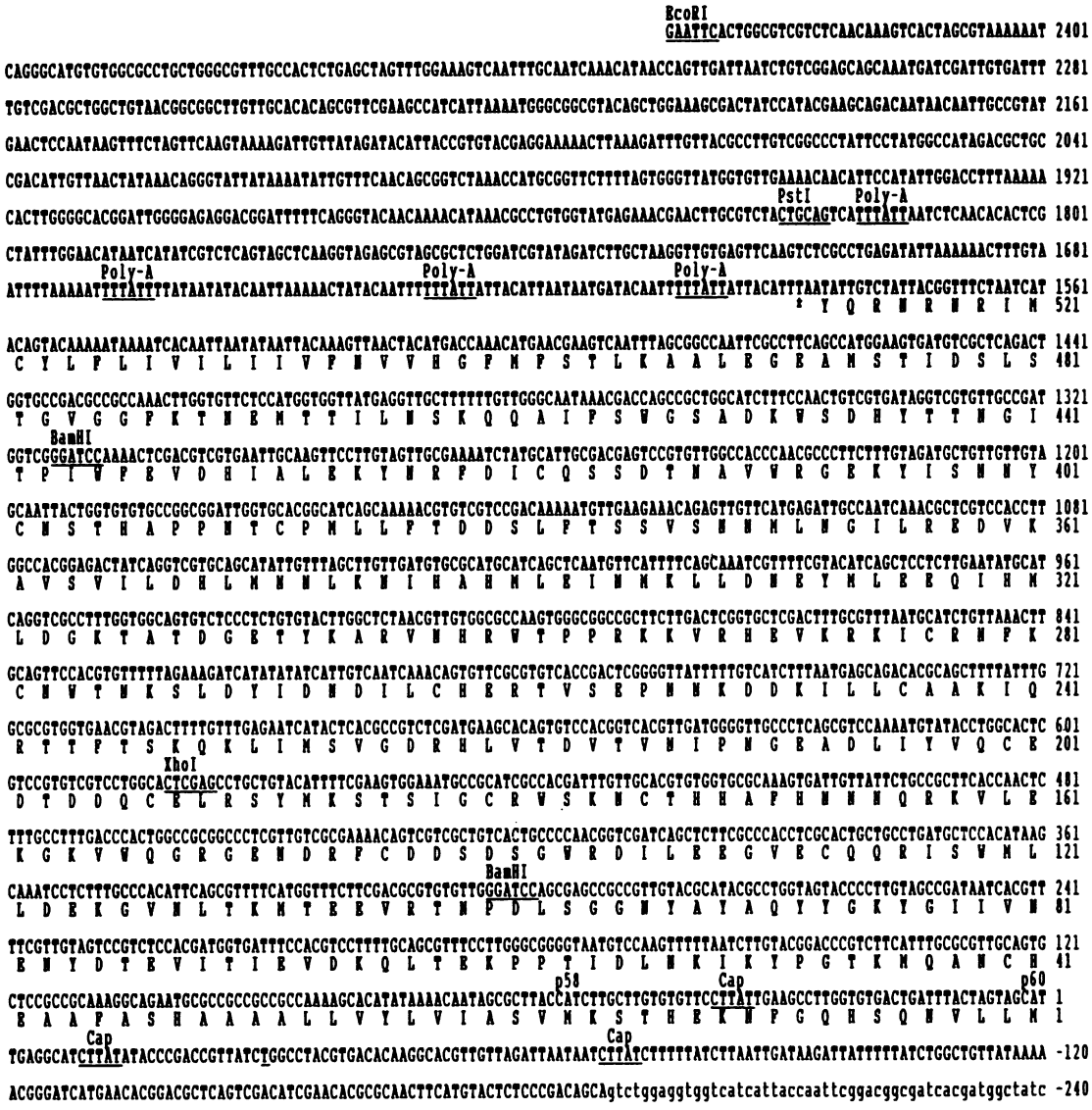

FIG. 5. Nucleotide sequence of the left end of *Eco*RI-H. The sequence is shown from left to right, relative to the standard Ac*M*NPV map (37) and extends 2,684 bases from the *Eco*RI site. Restriction sites are also shown for *Bam*HI, *Pst*I, and *Xho*I. p58 and p60 start sites, and potential baculovirus late mRNA cap sites (Cap) (46) are marked. Polyadenylation signals following the stop codon and complementary to AATAAA are indicated (Poly-A). Lowercase letters indicate a region of overlap with the sequence published by Oellig et al. (24).

genome (37) and is flanked by AT-rich regions (Fig. 4B). The stop codon at base 1588 is followed by four polyadenylation signals within the following 235 bases (Fig. 4B and 5). A second ATG at 54 nucleotides downstream from the +1 position is a potential translation initiation codon for a polypeptide of 511 amino acid residues (p58 in Fig. 5). There are six potential N-linked glycosylation sites within the p60 ORF and five within the p58 ORF (Fig. 4C). The p60 polypeptide encompasses two prominent hydrophobic clusters, shown in Fig. 4E, which we located near the N and C termini of p60. These are potential domains for an N-terminal signal peptide and a C-terminal membrane-spanning peptide.

A computer search for amino acid sequence homology with other known proteins showed no strong sequence homology of p60 with any other protein in the library (National Biomedical Research Foundation protein data library, March 1988 release).

DISCUSSION

A portion of the Ac*M*NPV genome that encodes gp67 has been located and sequenced. gp67 is a major virus envelope glycoprotein required for the penetration of virus into cells by adsorptive endocytosis (38, 39). Monoclonal antibodies reactive against the protein were used to screen a lambda gt11 expression library of Ac*M*NPV DNA fragments. DNA from the recombinants hybridized to the left end of *Eco*RI-H on the genome (Fig. 2). Sequence analysis (Fig. 5) revealed an ORF 1,590 nucleotides in length which could encode a polypeptide of 529 amino acid residues (molecular weight of 60,200). The ORF is transcribed from right to left and is flanked by AT-rich regions (Fig. 4E). Similar flanking regions have been observed with other baculovirus late and very-late genes (27). A nucleotide sequence from *Galleria mellonella* nuclear polyhedrosis virus (GmNPV) reported to contain an origin of DNA replication (1) was found to have

significant homology to the region of Ac*M*NPV DNA containing the gp67 ORF. The GmNPV sequence (1,016 nucleotides) lies entirely within the ORF of gp67 commencing at nucleotide 302 and exhibits near-perfect homology with the Ac*M*NPV sequence. The right-hand portion of the sequence given in Fig. 5 overlaps by 51 nucleotides a region of the genome at 81.2 to 85.0 mu sequenced by Oellig and colleagues (24) which contains several ORFs and a nested set of viral transcripts.

The sequence surrounding the first ATG found in the ORF (Fig. 5), TCAATGC, is considered less favorable for initiation of protein synthesis than that of a second ATG codon, which follows in the same reading frame at 54 nucleotides downstream where there is an A at −3 and a G at +4 (16) in the context AAGATGG. Kozak (15) reported that 5 to 10% of eucaryotic mRNAs examined have AUGs upstream from the known start site. The nontranslated AUG triplets occur in a context that is less favorable for translation initiation than the conserved pattern of nucleotides around the functional initiation codon. There are examples of animal virus proteins with two or more potential initiation codons in the same reading frame and in which both are used in the production of short and long forms of a polypeptide. An example is the thymidine kinase gene of herpes simplex virus type 1 which encodes two (and possibly three) polypeptides translated from a single mRNA using in-phase initiation codons (21). We have no data to indicate which initiation codon is used for translation of gp67 in insect cells.

Two sets of a consensus sequence (−ATAAG−) considered to be potential transcription start sites of baculovirus late and very-late genes (46) were found upstream of the p60 ORF (Cap in Fig. 5). A third consensus sequence was located between the p60 and p58 translation initiation codons. Future studies will probe the pattern of transcription in this portion of the genome.

A 2.1-kilobase transcript encoding the 67K glycoprotein was first detected at 2 h p.i. and was expressed most abundantly at about 12 h p.i. (Fig. 3). Stiles and Wood (33) detected a 67K virus-induced intracellular glycoprotein which appeared identical to the envelope glycoprotein gp67 as early as 2 h p.i. in infected cells. We observed that the transcript became less abundant in cells between 12 and 24 h p.i. and that two larger transcripts of 3.5 and 7.2 kilobases were seen at later time points (Fig. 3). Similar patterns of transcription have been described for the *Hin*dIII-I/*Eco*RI-F (I), *Hin*dIII-B_2/*Eco*RI-H (B_2) (19, 20), and *Hin*dIII-K/*Eco*RI-H,-S (K) (8) regions of the Ac*M*NPV genome. In these regions, nested transcripts with 3′ cotermini are transcribed with successive replacement of small early transcripts with larger late RNAs. It has been proposed that the larger RNAs arise from activation of upstream promoters and that coordinate suppression of downstream promoters by transcriptional interference or promoter occlusion results in the disappearance of the smaller transcripts (8).

The deduced amino acid sequence of gp67 (Fig. 5) has features in common with most membrane proteins. The hydropathy plot (Fig. 4E) reveals two hydrophobic regions, one located near the N terminus (amino acid residues 19 to 39) and a second near the C terminus. The N-terminal hydrophobic region may serve as a signal peptide having a 15-amino-acid core preceded by a basic amino acid (26, 44). The core is followed by a putative signal peptidase recognition sequence at residue 40 (45). However, amino-terminal sequencing of gp67 indicates that the signal peptide is cleaved two amino acids further at position 42 (Fig. 5), resulting in a 56.5K polypeptide (N. Sivasubramanian, Abstr. Annu. Meet. Soc. Invertebr. Pathol. 1988, 120, p. 103). The C-terminal hydrophobic region may act as the membrane anchor, having a putative 23-amino-acid membrane-spanning segment at residues 500 to 522 followed by a short tail of seven amino acids, three of which are basic arginines, and it may bind the viral nucleocapsid. This extension at the C terminus may be important in stabilizing virion structure.

gp67 was shown to contain N-linked carbohydrate moieties by failure to label the protein with *N*-acetylglucosamine in the presence of tunicamycin (41) and by endoglycosidase F digestion of purified virus (Fig. 1). Six potential N-linked glycosylation sites were found on the deduced amino acid sequence at positions 5, 177, 215, 372, 402, and 443 (Fig. 4C). However, the glycosylation site at position 5 is likely lost in the mature protein through removal of a signal peptide.

Some of the 15 cysteine residues in the polypeptide may be involved in intrachain folding (Fig. 4C). In addition, the cysteine near the C terminus at residue 520 of the protein may form a thioester with a fatty acid as found in envelope proteins of other viruses such as vesicular stomatitis virus (28). The acylated C terminus would likely help stabilize the anchorage of gp67 within the viral envelope.

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