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Proteins Encoded in the 81.2- to 85.0-Map-Unit Fragment of *Autographa californica* Nuclear Polyhedrosis Virus DNA Can Be Translated In Vitro and in *Spodoptera frugiperda* Cells

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We have previously demonstrated that five open reading frames exist in the nucleotide sequence of the 81.2- to 85.0-map-unit (m.u.) segment of plaque isolate E of *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA. The corresponding polypeptides are 9.8, 12.1, 36.6, 25.0, and 48.2 kDa in size (C. Oellig, B. Happ, T. Müller, and W. Doerfler, J. Virol. 63:1494, 1989), and we have investigated whether these proteins can be translated in infected cells. On subfragments of this viral DNA segment, mRNAs were selected from AcNPV-infected *Spodoptera frugiperda* insect cells at different times postinfection (p.i.). The in vitro translation of these RNAs in a rabbit reticulocyte-derived cell-free translation system yielded polypeptides of approximately 10 to 11, 12 to 14, 28, 36 to 38, and 48 to 50-kDa which were commensurate in size with the theoretically expected values. mRNAs for the 28- and 48- to 50-kDa proteins were identified by their translation products at 6 h p.i., and mRNAs for the 10- to 11-, 12- to 14-, and 36- to 38-kDa proteins were identified by their translation products at 12 h p.i. We constructed an AcNPV recombinant which carried in its polyhedrin gene the 3.9-kbp *Eco*RI-*Hin*dIII (81.8 to 84.8 m.u.) subfragment of the *Eco*RI J segment. Nucleotide sequence determinations revealed that the intact polyhedrin promoter lay adjacent to the additional 81.8- to 84.8-m.u. fragment in this recombinant. In *S. frugiperda* cells, which were infected with the recombinant AcNPV, a protein of 36 to 38 kDa was detected at 44 h p.i. in larger amounts than after infection with the nonrecombinant virus. However, there was no evidence for larger amounts of RNA derived from the 81.8- to 84.8-m.u. fragment in recombinant-infected cells. Recombinant-infected cells lacked the polyhedrin polypeptide. The synthesis of the 36- to 38-kDa polypeptide in recombinant- or AcNPV-E-infected *S. frugiperda* cells could be demonstrated by immunoprecipitation experiments. Peculiarly, this polypeptide was present in the cytoplasm as a 64-kDa glycoprotein. These data corroborate the notion that at least some of the open reading frames encoded in the 81.2- to 85.0-m.u. segment of AcNPV can be expressed in *S. frugiperda* cells.

The insect baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) offers the possibility of studying the regulation of viral genome expression in insect cells. The existence of multiple overlapping transcripts with common 3′ or 5′ termini has been described for the 32.6- to 41.0-, 81.8- to 86.4-, and 88.2- to 89.7-map-unit (m.u.) segments of AcNPV DNA (16), and has also been observed in many other parts of this viral genome (5, 19, 33). The evidence available to date renders it unlikely that RNA splicing plays a major role in the expression of the AcNPV genome in *Spodoptera frugiperda* insect cells (2, 15). It has been proposed that the sequential array of nonoverlapping reading frames in the AcNPV genome, combined with the transcriptional mode of nested sets of overlapping RNA transcripts, contributes to the regulated expression of viral genomes (16, 20).

To substantiate the occurrence of AcNPV-specific proteins, whose existence is suggested by the presence of open reading frames (ORFs) in the 81.2- to 85.0-m.u. segment of the plaque isolate E of AcNPV (AcNPV-E) genome (20, 30), RNAs from AcNPV-E-infected *S. frugiperda* cells were translated in vitro in a rabbit reticulocyte cell-free translation system.

In the present study, the AcNPV-*S. frugiperda* cell expression vector system (28) has been used for the expression of viral gene products which are encoded in the 81.2- to 85.0-m.u. segment of the AcNPV genome. Infection of *S. frugiperda* cells with the recombinant AcNPV yielded a viral protein of 36 to 38 kDa which could be immunoprecipitated with a mixture of different oligopeptide antibodies or with a monoclonal antibody directed against a polyhedral membrane component (7, 34, 35).

MATERIALS AND METHODS

Virus. In this investigation, AcNPV-E (30) was used. In the DNA of this isolate, a cellular DNA sequence of 634 nucleotides was inserted into the 81-m.u. segment (25). This cellular insert contained an additional *Eco*RI site. Hence the *Eco*RI restriction map of the DNA from this isolate (15, 20) was different from that of other isolates frequently used.

Standard techniques. The propagation of *S. frugiperda* cells and of AcNPV-E in culture (30), the preparation of AcNPV DNA and of the cloned *Eco*RI J fragment and its subfragments (15, 17, 20), the isolation and selection of mRNA (3, 4, 26), the labeling of DNA (21), and the cell-free translation protocol and the analysis of the in vitro-synthesized polypeptides (3, 4) or the dideoxy method of nucleotide sequence determinations (24) were described elsewhere. Synthetic oligodeoxyribonucleotides were synthesized in a model 381A DNA synthesizer (Applied Biosystems).

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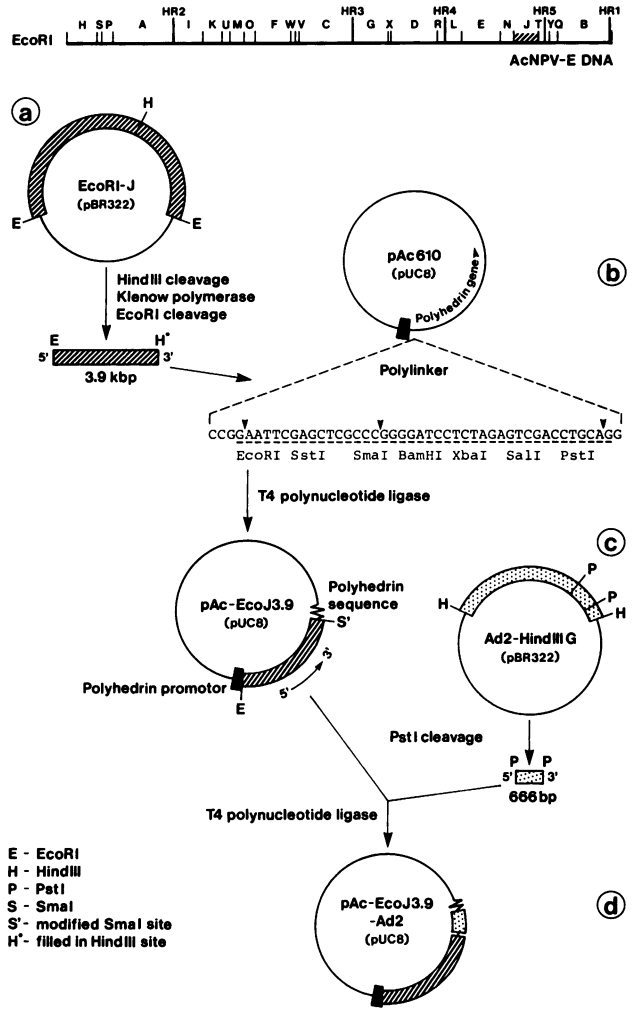


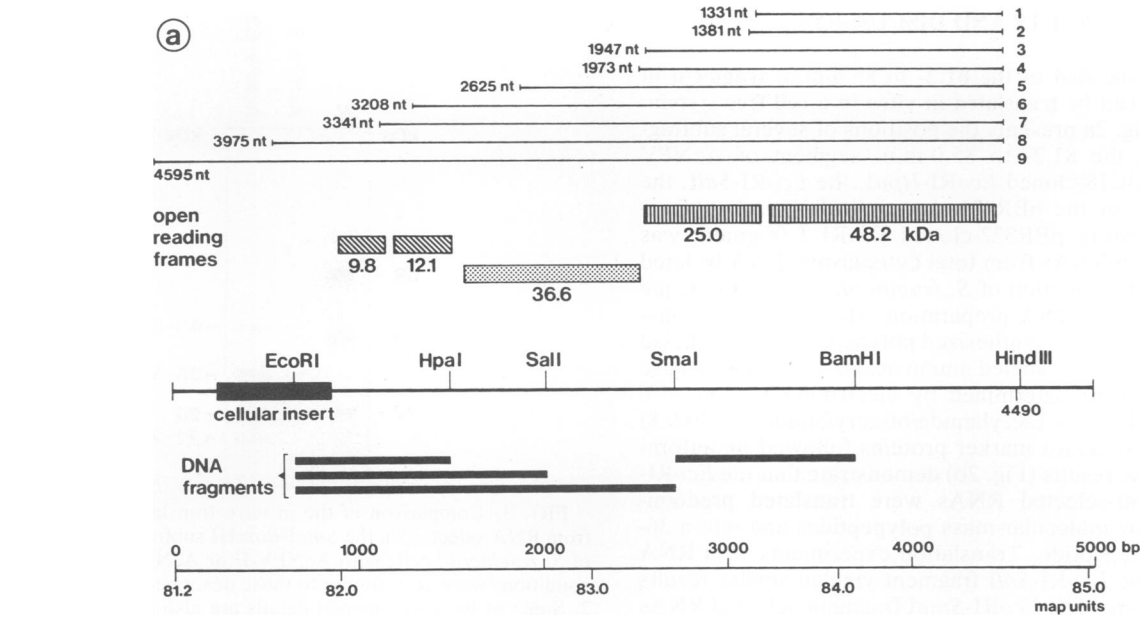
FIG. 1. Construction plan for the pAc610-derived recombinant that contained the 3.9-kbp *Eco*RI-*Hin*dIII (81.8 to 84.8 m.u.) fragment from the 81.2- to 85.0-m.u. segment of AcNPV-E DNA. The pBR322-cloned *Eco*RI J fragment was *Hin*dIII cleaved, and the generated ends were filled in with Klenow polymerase (11) and subsequently cut with *Eco*RI (a). The 3.9-kbp *Eco*RI (E)-blunted *Hin*dIII (H*) fragment was ligated into the *Eco*RI- and *Sma*I (blunt end)-cleaved polylinker segment of the pAc610 polyhedrin vector (b). The pBR322-cloned *Hin*dIII G fragment of Ad2 DNA (c) was cut with *Pst*I, and the 666-bp fragment between nucleotides 1835 and 2500 of Ad2 DNA (22) was ligated into the *Pst*I site in the polylinker segment of the pAc610 plasmid (18) which already carried the *Eco*RI-*Hin*dIII AcNPV DNA fragment. Thus, a pAc610 construct was generated which contained both the aforementioned AcNPV and Ad2 DNA fragments (d). This construct was used in cotransfection experiments of *S. frugiperda* cells with the DNA of AcNPV-E. From these transfection experiments, the recombinant plaques were isolated by using the inserted Ad2 DNA segment as a unique diagnostic hybridization target. At the top of the scheme, the *Eco*RI map of AcNPV-E DNA is shown with the *Eco*RI J fragment highlighted ▨.

Construction of recombinant AcNPV. The schemes in Fig. 1 outline the construction plan of a pAc610-based plasmid which contained in its polylinker site the *Eco*RI-*Hin*dIII fragment of the *Eco*RI J segment of AcNPV-E DNA and the 666-bp *Pst*I fragment of adenovirus type 2 (Ad2) DNA between nucleotides 1835 and 2500 (22). The inclusion of the Ad2 DNA fragment permitted the identification of recombinant plaques by hybridization (31) to ^{32}P-labeled Ad2 DNA. The presence of the additional *Eco*RI-*Hin*dIII fragment in the recombinants could not otherwise have been reliably monitored, because each AcNPV plaque already contained this sequence in the authentic position in the AcNPV genome. Recombinant AcNPV plaques were purified by three single-plaque isolations on *S. frugiperda* insect cells (8, 12). Virus stocks were generated on the same cells and used for further experiments. Recombinant AcNPV-infected, AcNPV-E-infected, or mock-infected cells were maintained in methionine-free medium starting 15 h postinfection (p.i.) or 15 h after mock infection, and at 20 h p.i. [^{35}S]methionine (30 μCi/ml; specific activity > 800 Ci/mmol; Amersham) was added. Proteins in the infected or mock-infected cells were analyzed 44 to 45 h p.i. by using a standard sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel, as described elsewhere (14).

Amplification of recombinant DNA by the polymerase chain reaction. The reaction mixture for polymerase chain reaction experiments contained 100 ng of recombinant AcNPV DNA, which carried the additional viral 81.8- to 84.8-m.u. fragment, 50 mM KCl, 10 mM Tris hydrochloride (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, the four deoxyribonucleoside triphosphates at 330 μM each, the two primers at 1 μM each (see the map in Fig. 4c), and 5 U of the *Taq* polymerase (23) in a total volume of 100 μl, which was overlaid with 75 μl of paraffin oil. The DNA was denatured at 95°C for 5 min and annealed at 60°C for 1 min, synthesis was allowed to proceed at 72°C for 1 min, and the reaction product was again denatured at 95°C for 1 min. This amplification cycle was repeated 25 to 30 times. In the final cycle, extension proceeded for 3 min. Subsequently, the paraffin oil was removed by chloroform extraction. The amplified DNA was purified by electrophoresis on a 1% low-melting-point agarose gel and by extraction with phenol and chloroform.

Immunization of rabbits with synthetic oligopeptides. From the amino acid sequence of ORF 3 encoded in the 81.2- to 85.0-m.u. fragment of AcNPV-E (see Fig. 5 in reference 20), two terminally and two internally located oligopeptides (see Fig. 6) were in vitro synthesized. The oligopeptides were a gift of Ronald Mertz, University of Munich, Munich, Germany. Rabbits were immunized by a standard procedure. Briefly, rabbits were injected once with 100 μg of the oligopeptide conjugated to bovine serum albumin or to keyhole limpet hemocyanin together with complete Freund adjuvant. Ten days after the initial injection, 50 μg of the conjugated oligopeptide was administered together with incomplete Freund adjuvant. This booster injection was repeated twice at 10-day intervals, and 10 days later the serum was collected. Preimmune sera were drawn prior to immunization.

In immunoprecipitation experiments, mixtures of antisera directed against the four different oligopeptides (see Fig. 7) and a modified procedure (10) were used. Briefly, 50 to 100 μl of [^{35}S]methionine-labeled cytoplasmic or nuclear extracts (ca. 1×10^6 labeled cells) was mixed with 10 μl of preimmune rabbit serum and incubated for 1 h at 4°C. Then, 50 μl of a 10% suspension of protein A-Sepharose CL-4B beads (Sigma) was added, and the mixture was incubated for another 30 min at 4°C. The nonspecifically bound proteins were removed by centrifugation, and 10 μl of each antipeptide serum was added to the supernatant. The mixture was incubated overnight at 4°C, and then 50 μl of a 10% protein A-Sepharose CL-4B bead suspension was added. The precipitate was collected by centrifugation after a 20-min incu-



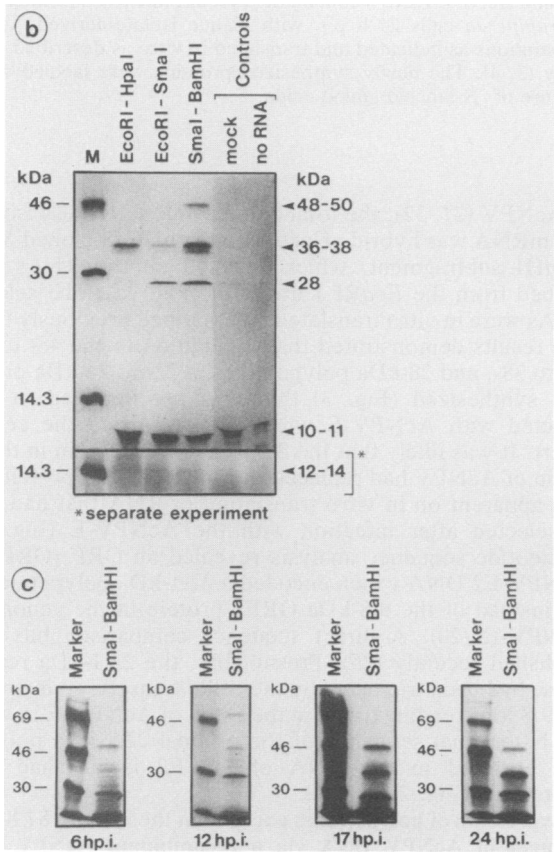


FIG. 2. Translation in a cell-free system of hybrid-selected RNAs isolated from *S. frugiperda* cells at 24 h after the infection with AcNPV-E in cell culture. (a) The transcriptional map of the 81.2- to 85.0-m.u. segment of the AcNPV genome reveals relevant restriction sites and the sizes and locations of the nine major overlapping RNA size classes transcribed from this segment (20). With modifications, this map was taken from reference 20. The bars above the restriction map (▨ , ▩ , ▥) designate the different reading frames for the potential polypeptides encoded in this region. The bars below the restriction map (▬) refer to the restriction endonuclease fragments that were used in hybrid selection experiments. The cellular insert in the AcNPV-E DNA was also designated (7, 20, 25). This sequence was derived from *S. frugiperda* cellular DNA (25). (b) Autoradiogram of a dried 15% polyacrylamide gel after the in vitro translation products were separated by electrophoresis. Most of the experimental conditions used are described in the text. The pUC18- or pBR322-cloned restriction subfragments were used to select specific RNA size classes (see panel a) and are shown at the top of the autoradiograms. Mock and no-RNA controls refer to in vitro translation experiments in which RNA from mock-infected *S. frugiperda* cells or no RNA at all was added to the rabbit reticulocyte lysates, respectively. As size marker (lane M), a mixture of ^{14}C-labeled proteins (Amersham) with molecular masses in kilodaltons, as indicated, was coelectrophoresed. The autoradiogram showing the 12- to 14-kDa protein was exposed three times longer than the other autoradiograms in this figure. (c) Time course of RNA synthesis in AcNPV-E infected cells. *S. frugiperda* cells were infected with AcNPV-E (30), and at 6, 12, 17 or 24 h p.i., as indicated, cytoplasmic RNA was isolated and hybrid selected on the *Sma*I-*Bam*HI subfragment of the m.u. 81.2 to 85.0 segment of the AcNPV genome. The *Sma*I-*Bam*HI subfragment selects all size classes of RNAs transcribed from the 81.2- to 85.0-m.u. segment (see panel a). In vitro translation and analyses of the polypeptides synthesized in the cell-free system proceeded as described in the text. Proteins were labeled by addition of a mixture of ^{3}H-labeled amino acids to the reaction mixture. The sizes of the coelectrophoresed marker proteins (in kilodaltons) are indicated.

bation and washed twice with NET-I buffer (50 mM Tris hydrochloride [pH 7.4], 0.15 M NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 2 mg of ovalbumin per ml) and twice with NET-II buffer (NET-I buffer with 0.5 M NaCl). The immunoprecipitated proteins were released from the beads by heating the sample to 100°C for 10 min in sample buffer (62.5 mM Tris hydrochloride [pH 6.8], 7 M urea, 2% SDS, 5% β-mercaptoethanol, 10% glycerol). The beads were removed by centrifugation, and the proteins were analyzed by electrophoresis on 12.5% gels containing SDS. For immunoprecipitation with the monoclonal antibody MAB34F2 (34) (10 μl), the protein extract was preincubated and precipitated with 50 μl of an anti-mouse antibody–Sepharose CL-4B bead suspension.

RESULTS AND DISCUSSION

The ORFs encoded in the 81.2- to 85.0-m.u. fragment of AcNPV DNA can be translated in vitro in a cell-free system. The map in Fig. 2a presents the positions of several subfragments within the 81.2- to 85.0-m.u. segment of AcNPV DNA. The pUC18-cloned *Eco*RI-*Hpa*I, the *Eco*RI-*Sal*I, the *Sma*I-*Bam*HI, or the pBR322-cloned *Eco*RI-*Sma*I subfragment or the entire pBR322-cloned *Eco*RI J fragment was used to select mRNAs from total cytoplasmic RNA isolated at 24 h after the infection of *S. frugiperda* cells with plaque isolate AcNPV-E. RNA preparations (Fig. 2b) were translated, and the in vitro-synthesized polypeptides were labeled with a mixture of ^{3}H-labeled amino acids. The sizes of these polypeptides were determined by electrophoresis on 15% polyacrylamide gels (acrylamide/bisacrylamide = 30:0.8) and by using standard marker proteins followed by autoradiography. The results (Fig. 2b) demonstrate that the *Eco*RI-*Hpa*I fragment-selected RNAs were translated predominantly into low-molecular-mass polypeptides and into a 36- to 38-kDa polypeptide. Translation experiments with RNA selected on the *Eco*RI-*Sal*I fragment yielded similar results (data not shown). The *Eco*RI-*Sma*I fragment-selected RNAs were translated into proteins of 10 to 11, 12 to 14, 28, and 36 to 38 kDa. The 10- to 11-kDa polypeptide migrated close to the front in this 15% polyacrylamide gel system. The translation patterns (data not shown) obtained with RNAs isolated on the *Sma*I-*Bam*HI fragment (Fig. 2b) or the EcoRI J fragment did not differ, since the same RNA molecules were selected on this subfragment as on the entire *Eco*RI J fragment (map in Fig. 2a). On these latter DNA fragments, we hybrid selected RNAs that encompassed the entire population of RNAs 1 to 9 which had been mapped previously (20) in this viral DNA segment (Fig. 2a). These translational patterns and the sizes of individual polypeptides (10 to 11, 12 to 14, 28, 36 to 38, and 48 to 50 kDa) were consistent with the assignments of the map locations for the individual ORFs which had been determined by nucleotide sequence analyses (20). In particular, for the higher-molecular-mass RNAs, the ORFs encoded in the 5′-located sequences of these RNAs were preferentially translated, whereas the more 3′-encoded polypeptides were less efficiently translated or their syntheses could not readily be detected.

We next determined the time course for the synthesis of individual RNA size classes that were transcribed off the EcoRI J fragment of AcNPV DNA at various times after infection. These RNAs were hybrid selected on the *Sma*I-*Bam*HI fragment (Fig. 2a) for in vitro translation. The results of the corresponding analyses on polyacrylamide gels, which were illustrated by autoradiography (Fig. 2c), revealed that the mRNAs encoding the 28- and 48- to 50-kDa proteins were synthesized 6 h p.i. At 12 h p.i., mRNAs translatable into the smaller polypeptides (data not shown) and into 28, 36- to 38-, and 48- to 50-kDa proteins were apparent (Fig. 2c). At 24 h p.i., the 48- to 50-kDa protein decreased in intensity. These findings indicated that the higher-molecular-mass RNAs (Fig. 2a) were synthesized starting at about 12 h p.i. With mRNAs isolated at later times after infection (17, 24 h p.i.), all size classes of proteins were translated.

Since the AcNPV-E variant with a cellular insert (Fig. 2a) (20, 25) was used in all of the experiments reported here, it was necessary to investigate AcNPV-specific RNAs from the 81.2- to 85.0-m.u. segment after infection of *S. frugiperda* cells with an AcNPV strain devoid of this insert. At 24 h after infection of *S. frugiperda* cells with the E2 strain

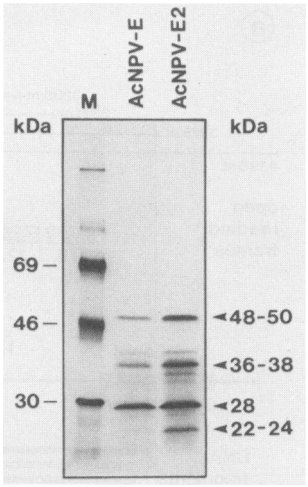


FIG. 3. Comparison of the in vitro translation patterns derived from RNA selected on the *Sma*I-*Bam*HI subfragment after infection of *S. frugiperda* cells with AcNPV-E or AcNPV-E2. Experimental conditions were very similar to those described in the legend to Fig. 2. Some of the experimental details are also described in the text. Lane M contains ^{14}C-labeled marker polypeptides. Lanes AcNPV-E (30) and AcNPV-E2 (27) contain the cytoplasmic RNA isolated from *S. frugiperda* cells 24 h p.i. with plaque isolate-derived AcNPV preparations as indicated and translated in vitro as described previously (3, 4). The newly synthesized proteins were labeled with a mixture of ^{3}H-labeled amino acids.

of AcNPV (27, 32), the total cytoplasmic RNA was isolated and mRNA was hybrid-selected on the pUC18-cloned *Sma*I-*Bam*HI subfragment, which selected all the RNAs transcribed from the *Eco*RI J fragment (Fig. 2a). The selected RNAs were in vitro translated as described previously (3, 4). The results demonstrated that in addition to the 48- to 50-, 36- to 38-, and 28-kDa polypeptides, a 22- to 24-kDa protein was synthesized (Fig. 3) that was not detected in cells infected with AcNPV-E whose DNA carried the cellular insert. It was likely that the 22- to 24-kDA protein in the E2 strain of AcNPV had replaced the 10- to 11-kDa protein that was apparent on in vitro translation of RNA that had been preselected after infection with the AcNPV-E (Fig. 2b). Nucleotide sequence analysis revealed an ORF (ORF1) in AcNPV-E2 DNA which encoded a 22.1-kDa polypeptide (7, 20) instead of the 9.8-kDa ORF1 protein in the genome of AcNPV-E (20). A direct sequence comparison has been published recently (25). Presumably, the 22.1-kDa reading frame had been truncated by the cellular insert, giving rise to the 9.8-kDa reading frame in the DNA of AcNPV-E in which the N-terminal sequence of the original 22.1-kDa polypeptide encoded in the DNA of the E2 isolate had been obliterated (Fig. 2a).

Expression of polypeptides encoded in the 81.8- to 84.8-m.u. segment of AcNPV DNA via a recombinant AcNPV in *S. frugiperda* cells. It was reasoned that the polypeptides encoded in the 81.8- to 84.8-m.u. segment of AcNPV DNA might be synthesized in *S. frugiperda* cells at an elevated level when an additional copy of this segment was introduced into recombinant AcNPV in close vicinity to its polyhedrin promoter. The construction of this recombinant was illustrated in Fig. 1. The results of experiments in which restriction endonuclease (*Eco*RI, *Eco*RI-*Pst*I, and *Hin*dIII) and Southern blot hybridiziation (29) analyses were per-

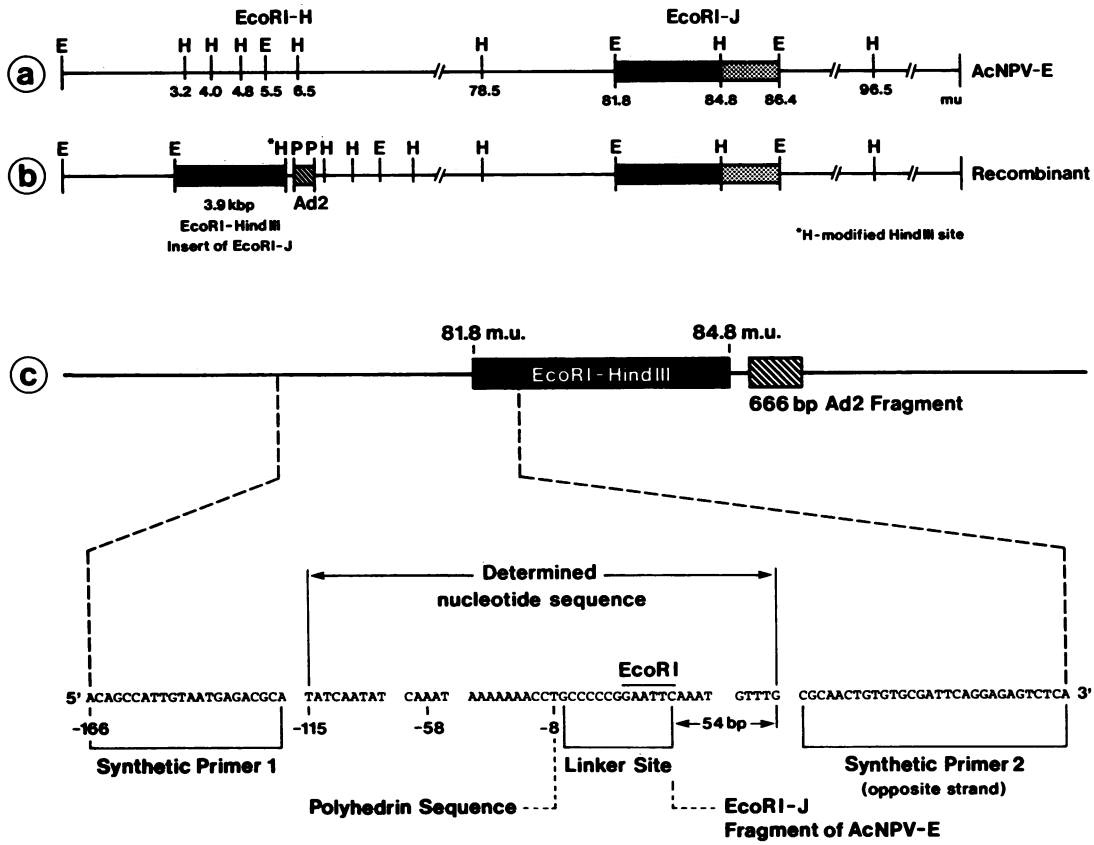


FIG. 4. Restriction and nucleotide sequence analyses of an AcNPV recombinant that contained in the EcoRI H fragment of its DNA an additional *Eco*RI-*Hin*dIII fragment, the 81.8- to 84.8-m.u. segment of AcNPV DNA, and a 666-bp Ad2 DNA tracer segment. (a and b) Maps showing parts of the AcNPV-E genome (a) and of the recombinant genome containing the additional copy of the *Eco*RI-*Hin*dIII fragment from the *Eco*RI J region (b). (c) Nucleotide sequence across the junction between the polyhedrin promoter and the inserted 81.8- to 84.8-m.u. segment from the *Eco*RI J fragment in the genome of the AcNPV recombinant. Details are described in the text. The sequences of the synthetic oligodeoxyribonucleotide primers are indicated. The schematic drawing presents some of the sequence elements in the recombinant and the location of the junction via an *Eco*RI linker site. The numbers −8, −58, etc., refer to nucleotide numbers in the authentic polyhedrin sequence (9).

formed demonstrated that the authentic *Eco*RI H or the corresponding *Hin*dIII F fragment in the DNA of AcNPV-E disappeared and that two new restriction fragments were generated (data not shown; maps of the AcNPV-E and recombinant virus are shown in Fig. 4a and b, respectively).

The nucleotide sequence at the site of linkage between the vector-derived polyhedrin promoter and the inserted *Eco*RI-*Hin*dIII fragment (81.8 to 84.8 m.u.) in the recombinant AcNPV DNA was determined by using as primers synthetic oligodeoxyribonucleotides whose position in the sequence was apparent from Fig. 4c. A nucleotide segment lying between the two primers (Fig. 4c, synthetic primers 1 and 2) was amplified by the polymerase chain reaction with *Taq* polymerase and was then sequenced starting from primer 1. A comparison of the nucleotide sequence shown in Fig. 4c with the published sequence data for the polyhedrin promoter (9) and for the *Eco*RI J fragment (20) of AcNPV-E revealed that the two authentic sequences were preserved intact and fused to each other. Hence, at least theoretically, the additional 81.8- to 84.8-m.u. fragment that was recombined into the *Eco*RI H fragment could have been transcribed under the control of the fused polyhedrin promoter. Nucleotide 676 from the *Eco*RI N/J region of AcNPV-E (Fig. 2) (20) was fused via the *Eco*RI polylinker region to nucleotide −8 of the polyhedrin promoter sequence (9) (Fig. 4c).

It was of interest to determine whether the additional 81.8- to 84.8-m.u. segment of AcNPV DNA, which was inserted into the *Eco*RI H fragment in the recombinant virus, was transcribed at all, whether it was under its own promoter control, or whether this control was dominated by the adjacent polyhedrin promoter (map in Fig. 1). *S. frugiperda* cells growing in monolayer cultures were infected with AcNPV-E or with the recombinant AcNPV containing the small Ad2 DNA segment and the additional 81.8- to 84.8-m.u. fragment of AcNPV DNA. The cytoplasmic RNA was isolated at 24 h p.i. RNA transfer (Northern blot) and hybridization analyses with the cloned, ^{32}P-labeled *Eco*RI J fragment as probe revealed the presence of identical RNA size classes and similar amounts of *Eco*RI J fragment-specific RNA in both the total cytoplasmic and the poly(A)$^+$-selected (1) RNA preparations from cells infected with AcNPV or the recombinant virus (data not shown). The size classes observed were similar to those described earlier for AcNPV-E-infected *S. frugiperda* cells (16, 20).

Cytoplasmic RNA from recombinant-infected *S. frugiperda* cells was then isolated at 24 h p.i. and subjected to

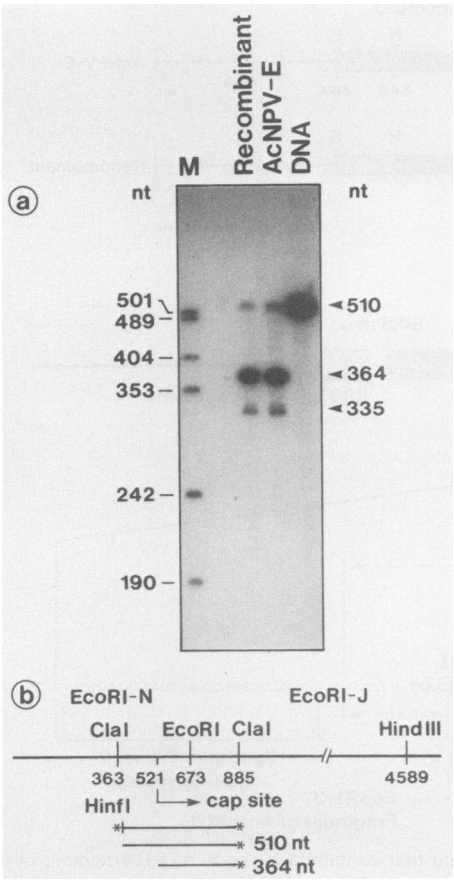


FIG. 5. S1 nuclease protection analysis of an *Eco*RI J promoter fragment by cytoplasmic RNA isolated from recombinant AcNPV- or AcNPV-E infected *S. frugiperda* cells. Most of the experimental conditions are described in the text. (a) Autoradiogram of a 5% polyacrylamide–7 M urea gel on which protected DNA fragments were resolved by electrophoresis. The hybridization probe was a 510-nucleotide (nt) *Hin*fI-*Cla*I fragment whose map location is designated in panel b. This fragment was 5′ end labeled with polynucleotide kinase and [γ-^{32}P]ATP and hybridized at 43°C under standard conditions (20) to cytoplasmic RNA from *S. frugiperda* cells which were infected with virus as marked in the graph in panel a. The lane DNA indicated that the probe was not hybridized to RNA, but alkali treated and coelectrophoresed. pUC18 DNA was *Hpa*I cut, and the fragments were 5′ end labeled as described above, alkali denatured, and coelectrophoresed as marker (lane M). (b) Partial map of the 81.2- to 85.0-m.u. (*Eco*RI-N and -J) fragment of which the *Eco*RI-*Hin*dIII segment (81.8 to 84.8 m.u.) had been inserted into the pAc610 plasmid (Fig. 1). The location of the *Hin*fI-*Cla*I fragment probe is indicated. The probe was ^{32}P-labeled (*) at one end. The size of the marker fragments is designated in nucleotides. The cap site of RNA 8 (20) is marked by an arrow.

S1 nuclease protection analysis by using the 510-nucleotide *Hin*fI-*Cla*I fragment (Fig. 5b) as the hybridization probe. Experimental conditions were those described elsewhere (20) and outlined in the legend to Fig. 5. After S1 nuclease treatment of the DNA-RNA hybrids, a ^{32}P-labeled DNA fragment of 364 nucleotides was protected; this fragment length was identical in AcNPV-E-infected and in recombinant-infected *S. frugiperda* cells (Fig. 5a). The presence of a weaker 335-nucleotide band suggested the existence of a second start site.

The occurrence of the expected 364-nucleotide fragment in S1 protection experiments indicated that the transcribed RNA was derived only from one or both 81.8- to 84.8-m.u. regions in the recombinant AcNPV genome. Had the fused polyhedrin promoter served as the site of initiation with the authentic polyhedrin cap site (Fig. 5a), a fragment of 270 nucleotides (212 nucleotides [*Eco*RI-*Cla*I; Fig. 5b] plus 58 nucleotides [cap site of polyhedrin to site of junction; Fig. 4c]) would have been protected. A fragment of this size was not apparent (Fig. 5a). Thus, there was no evidence for the transcription of the additional copy of the 81.8- to 84.8-m.u. fragment under the control of the polyhedrin promoter.

Immunoprecipitation of a specific 36- to 38-kDa polypeptide. For some of the immunoprecipitation experiments

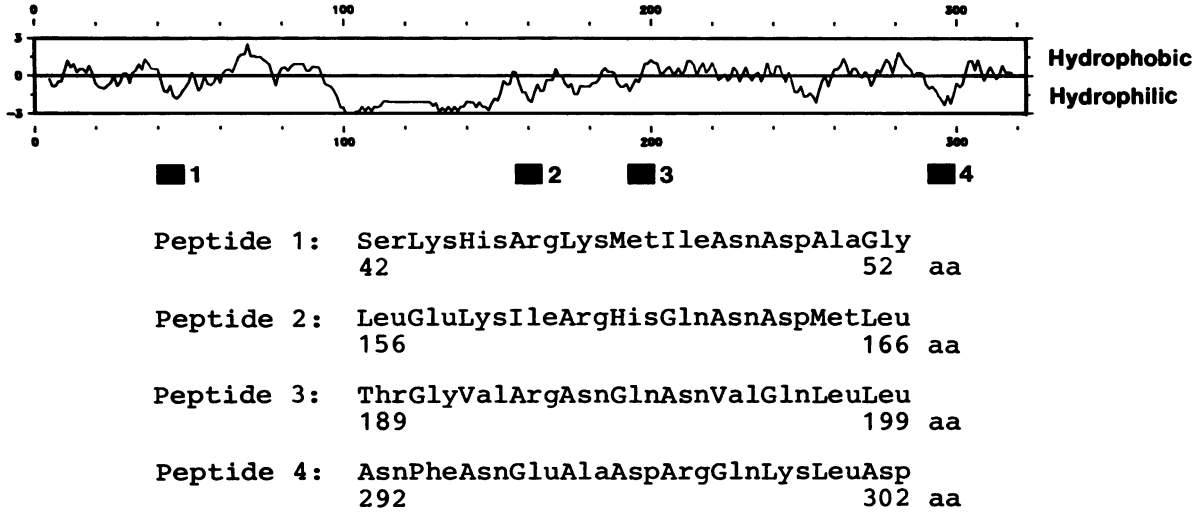


FIG. 6. Map locations and amino acid (aa) sequences of four undecapeptides in ORF3 which were used to raise monospecific antibodies. The amino acid sequence of ORF3 in the 81.2- to 85.0-m.u. region of AcNPV-E DNA was published previously (20). The hydropathy plot of the ORF3 polypeptide and the locations and sequences of undecapeptides 1 to 4 are presented here.

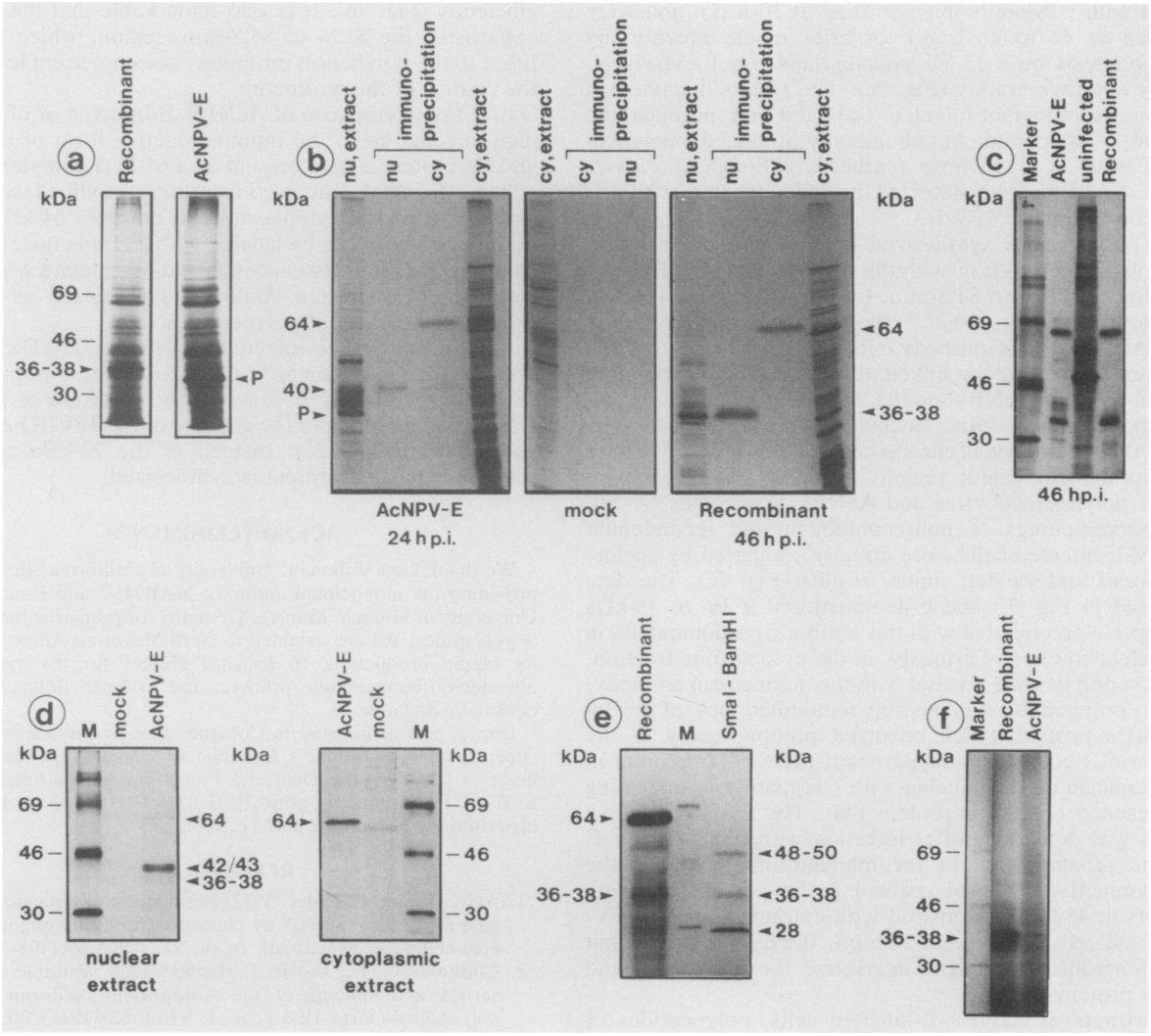


FIG. 7. Expression of the ORF3 36- to 38-kDa protein in recombinant- or AcNPV-E-infected *S. frugiperda* cells or in in vitro translation experiments. (a) Autoradiogram of an SDS–12.5% polyacrylamide gel on which proteins were resolved that had been synthesized in the presence of [^{35}S]methionine in AcNPV-E-infected or recombinant-infected *S. frugiperda* cells. In these experiments, [^{35}S]methionine was added 20 h p.i. and extracts were prepared at 44 to 46 h p.i. The gel positions of the standard ^{14}C-labeled marker proteins, as well as the positions of polyhedrin (P) (in AcNPV-E-infected cells) and of the 36- to 38-kDa protein, derived from ORF3 in the 81.2- to 85.0-m.u. segment of AcNPV DNA (in recombinant-infected cells), are marked. Experimental conditions were described in the text. (b and c) *S. frugiperda* cells were infected with AcNPV-E, mock infected, or infected with the recombinant that contained an additional 81.8- to 84.8-m.u. fragment of the AcNPV-E genome. At 15 h p.i. [^{35}S]methionine (30 μCi/ml) was added to the maintenance medium, and at 24 or 46 h p.i. (AcNPV-E) or 46 h p.i. (recombinant AcNPV) the nuclear (nu) or cytoplasmic (cy) fractions (panel b) or total cell extracts (panel c) were prepared. The total extracts were electrophoresed or specific proteins were immunoprecipitated with the monoclonal antibody MAB34F2 (34) prior to electrophoretic separation on an SDS–12.5% polyacrylamide gel. (d) Experimental conditions were very similar to those described in panels b and c, except that in the immunoprecipitation reaction a mixture of the monospecific sera raised against the four different undecapeptides (Fig. 6) was used. (e) The same antibodies were used to precipitate specific proteins from extracts of recombinant AcNPV-infected cells. As a control, the in vitro translation reaction products, whose synthesis was stimulated by RNA preselected on the *Sma*I-*Bam*HI fraction of the *Eco*RI J fragment of AcNPV-E, were coelectrophoresed. (f) AcNPV-E-infected or recombinant-infected cells were labeled with $^{32}P_i$ (50 μCi/ml) from 28 to 52 h p.i. Subsequently, whole-cell extracts were electrophoresed on an SDS–12.5% polyacrylamide gel.

described in this report, a mixture of four different monospecific antisera was used. These sera were raised against four different undecapeptides located in four different domains in the ORF3 polypeptide in the 81.2- to 85.0-m.u. region of AcNPV DNA (20). This protein was chosen for further investigations because of the interesting arginine-serine repeats in its amino acid sequence (20). The hydropathy plot (13) of the ORF3 polypeptide is presented in Fig. 6, which also shows the locations and amino acid sequences of the four synthetic undecapeptides (peptides 1 to 4).

It was the aim of this study to investigate whether the polypeptides encoded in the 81.2- to 85.0-m.u. segment of AcNPV DNA were actually expressed in infected *S. frugiperda* cells. As described in Materials and Methods, the polypeptides synthesized in mock-infected, AcNPV-E-infected, or recombinant-infected *S. frugiperda* cells were

labeled with [^{35}S]methionine starting at 20 h p.i. and were analyzed at 44 to 46 h p.i. or after mock infection by electrophoresis on a 12.5% polyacrylamide gel and visualized by autoradiography (Fig. 7a). The results documented that the recombinant-infected cells did not produce the polyhedrin polypeptide but abundantly produced a polypeptide of 36 to 38 kDa whose synthesis, although to a lesser extent, could also be detected in cells infected with the nonrecombinant AcNPV-E.

The polypeptides synthesized in *S. frugiperda* cells infected with AcNPV-E or with the recombinant AcNPV with an additional 81.8- to 84.8-m.u. fragment were analyzed by immunoprecipitation with the monoclonal antibody MAB34F2 (34). This antibody exhibited specificity toward a phosphorylated protein linked to the carbohydrate layer surrounding the AcNPV or the *Orgyia pseudotsugata* nuclear polyhedrosis virus occlusion bodies (7, 34). The MAB34F2 antibody was chosen because of sequence homologies in the equivalent regions of the *O. pseudotsugata* nuclear polyhedrosis virus and AcNPV-E genomes (7, 20). Immunoprecipitates of nonrecombinant and recombinant AcNPV-E-infected cells were directly compared by coelectrophoresis and yielded similar results (Fig. 7c). The data presented in Fig. 7b and c demonstrated a 36- to 38-kDa polypeptide precipitable with this antibody predominantly in the nuclear fraction. Curiously, in the cytoplasmic fraction, a 64-kDa polypeptide reacted with the monoclonal antibody, and this polypeptide was possibly a modified form of the 36- to 38-kDa protein which occurred predominantly in the cytoplasm. Recent data suggest that the 64-kDa protein in the cytoplasm can be labeled with [^{3}H]mannose, indicating the presence of a glycoprotein (7a). The immunoreactive protein was detectable after infection with both AcNPV-E and the recombinant. In recombinant-infected cells, the immunoreactive material seemed to be present in larger amounts at 46 h p.i. compared with extracts from AcNPV-E-infected cells analyzed at 24 h p.i. We do not know what sort of modification might interrelate the 36- to 38- and 64-kDa proteins.

In extracts of AcNPV-E-infected cells, polypeptides of similar sizes were also precipitated (Fig. 7d) with a mixture of the four different monospecific antibodies raised against four undecapeptides in ORF3 in the *Eco*RI J fragment of AcNPV-E (Fig. 6). After the infection of *S. frugiperda* cells with recombinant AcNPV, the 36- to 38- and 64-kDa immunoprecipitates were detectable (Fig. 7e) by the reaction with a mixture of antibodies against the four undecapeptides. Lastly, the results presented in Fig. 7f demonstrated that the 36- to 38-kDa protein, which was overexpressed in recombinant AcNPV-infected compared with AcNPV-E-infected cells, was a phosphoprotein.

The results summarized in this section are consistent with the interpretation that the 36- to 38-kDa ORF3 polypeptide encoded in the *Eco*RI J fragment of AcNPV-E is probably identical with the polyhedrin-associated calyx protein previously described (7, 34, 35).

Moreover, the data presented here offer a number of interesting observations which will require further analyses. (i) The recombinant AcNPV genome carries the region of 81.8 to 84.8 genome units twice. There is no evidence from the results of S1 nuclease protection experiments that recombinant virus-infected cells would produce more stable RNA specific for this viral genome segment than would nonrecombinant virus-infected cells at 24 h p.i. Nevertheless, recombinant virus-infected cells overproduce the 36- to 38-kDa protein, suggesting that the RNA is translated more efficiently (Fig. 7b). It is also remarkable that the fragment comprising the 81.8- to 84.8-m.u. region, which has been fused to the polyhedrin promoter, does not seem to be under the control of this promoter.

(ii) In the cytoplasm of AcNPV-E-infected or of recombinant-infected cells, the immunoreactive form of the 36- to 38-kDa protein is also present as a 64-kDa complex (Fig. 7b and c). In what way is this protein modified when it is synthesized in the cytoplasm? This complex 64-kDa protein in the cytoplasm can be labeled with [^{3}H]mannose at 24 and 46 h p.i. (7a). It is also necessary to investigate whether the 36- to 38-kDa protein could be synthesized as a fusion product with adjacent polypeptides.

(iii) In AcNPV-E-infected cells the 22-kDa protein, present after infection with the E2 isolate, is not produced (Fig. 3), presumably because of the insertion of a cellular DNA segment (25) into the genome of AcNPV-E between 81 and 82 m.u. (Fig. 2a). Instead of the 22-kDa protein, a 9.8-kDa truncated protein is synthesized.

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