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Hybridization Selection and In Vitro Translation of *Autographa californica* Nuclear Polyhedrosis Virus mRNA

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We isolated polyadenylated RNA from the cytoplasm of cells infected with *Autographa californica* nuclear polyhedrosis virus late after infection (21 h postinfection). At that time intracellular protein synthesis was directed almost exclusively toward infected cell-specific proteins. The polyadenylic acid-containing RNA sequences in the cytoplasm at 21 h postinfection were radiolabeled in vitro and hybridized to *A. californica* nuclear polyhedrosis virus DNA restriction fragments. The polyadenylic acid-containing RNA was derived from regions representing the entire viral genome. Translation in a reticulocyte cell-free protein-synthesizing system of cytoplasmic RNA selected by hybridization to viral DNA and polyadenylic acid-containing RNA produced almost identical polypeptide patterns, suggesting that late after infection almost all of the cytoplasmic polyadenylic acid-containing RNA present in infected cells was of viral origin. Polyhedrin protein (molecular weight, 33,000) and a number of virion structural proteins were among the translation products which were identified by immunoprecipitation and by comparing molecular weights. In addition, some tentative nonstructural infected cell-specific proteins were also detected. Using the hybridization selection technique, we determined that sequences complementary to the message coding for polyhedrin were located on *Eco*RI fragment I of *A. californica* nuclear polyhedrosis virus DNA, whereas sequences coding for a putative nonstructural protein (molecular weight, 39,000) were on *Eco*RI fragment J.

Nuclear polyhedrosis viruses are members of subgroup A in the family Baculoviridae. A distinctive feature of these invertebrate DNA viruses is the occlusion of many enveloped virus particles in a large paracrystalline inclusion, which is referred to as a polyhedron. About 95% of the polyhedron mass is composed of a single polypeptide having a molecular weight of approximately 30,000, which is referred to as polyhedrin.

The multiply embeded nuclear polyhedrosis virus of the alfalfa looper *Autographa californica* (AcMNPV) has been studied most because of its ability to replicate efficiently in a number of insect cell lines. However, the biochemical events involved in DNA replication, RNA transcription, and protein synthesis in this and other baculoviruses have been studied very little. The synthesis of infected cell-specific proteins (ICSPs) starts at about 4 h postinfection (p.i); with time, both the number and the relative amount of ICSPs increase, whereas host cell protein synthesis decreases (5, 9, 14, 32). A number of ICSPs at late times after infection have molecular weights similar to the molecular weights of virion structural polypeptides. Polyhedrin is detected as early as 12 h p.i., but its synthesis is predominant only at late times after infection (14). The experiments described below were part of a larger study, the ultimate aim of which is the identification of viral RNA transcripts and the locations of genes for ICSPs, including virus structural proteins and polyhedrin, on the genome of AcMNPV.

Recently, there has been direct evidence that polyhedrin is coded for by the viral genome. Upon mixed infection by plaque-purified AcMNPV and *Rachiplusia ou* multiply embedded nuclear polyhedrosis virus, the distinctive polyhedrins of these viruses segregated into recombinant viruses (23). In addition, virus-specific RNA was isolated from AcMNPV-infected cells and translated in vitro into polyhedrin (27). The precise location of the polyhedrin gene on the DNA of AcMNPV is not yet known. However, recombination studies have shown that the approximate location of the coding sequence for polyhedrin is between map positions 0.70 and 0.89 on the AcMNPV genome, and it has been hypothesized that the polyhedrin gene is most

likely located in *Eco*RI fragment I of AcMNPV DNA (23).

In this paper we show that polyadenylic acid-containing [poly(A)$^+$] mRNA's that were present late in infection hybridized asymmetrically to regions along the entire genome. We also describe in vitro translation experiments with RNA selected by hybridization to AcMNPV DNA and to some purified *Eco*RI restriction fragments. This strategy is often used to provide information concerning the origins and locations of mRNA's and gene functions on viral genomes (6, 7, 9, 12).

MATERIALS AND METHODS

Cells and virus. We used the continuous cell line *Spodoptera frugiperda* IPLB-SF21 (28), which was supplied by D. L. Knudson. The cells were grown at 27°C in Grace insect tissue culture medium (KC Biologicals) supplemented with crystallized bovine albumin (5.55 g/liter; Pentex) TC-yeastolate (3.33 g/liter; Difco Laboratories), lactalbumin hydrolysate (3.33 g/liter; Difco), and 10% heat-inactivated fetal bovine serum. The cells were maintained in spinner cultures, and 4×10^7 log-phase cells were seeded into plastic tissue culture flasks (150 cm^2) before inoculation with virus.

The plaque-purified variant E2 of AcMNPV (18) was used to infect cells at a multiplicity of 10 PFU per cell. This multiplicity resulted in an apparently synchronous infection since polyhedra appeared in most cells at about 18 h p.i.

Isolation of viral DNA. AcMNPV DNA was purified from virions isolated from polyhedra (18). The DNA was digested with restriction endonucleases (Bethesda Research Laboratories or Boehringer Mannheim Corp.) and analyzed as described previously (29). The DNA fragments were designated by the method of Smith and Summers (18). *Eco*RI fragments I and J were purified by two cycles of agarose gel electrophoresis, as described previously (19). In the experiment shown in Fig. 1, *Eco*RI fragments I (lane c) and J (lane d) were analyzed on a 0.8% agarose gel and compared with an *Eco*RI digest of AcMNPV DNA (lane b). Contamination of *Eco*RI fragments I and J with other DNA fragments was not revealed by staining the gels with ethidium bromide.

RNA isolation. AcMNPV-infected cells were harvested at 21 h p.i. and washed with phosphate-buffered saline. The cells were lysed in 30 mM Trishydrochloride (pH 7.5)–10 mM magnesium acetate–1% Nonidet P-40 for 30 min on ice. The nuclei were sedimented by centrifugation at 1,500 × *g* and 4°C for 10 min, and the resulting supernatant was centrifuged at 10,000 × *g* and 4°C for 10 min. The 10,000-×-*g* (postmitochondrial) supernatant was extracted twice with phenol, saturated with 10 mM Tris-hydrochloride (pH 7.5)–0.1 M NaCl–10 mM EDTA, and extracted once with chloroform-isoamyl alcohol (24:1). The phenol-chloroform phase was extracted once with buffer, and the combined aqueous phases were extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The RNA was precipitated with 66% ethanol containing 0.2 M potassium acetate overnight at −20°C. About 800 μg of postmitochondrial RNA was obtained from 5×10^7 cells.

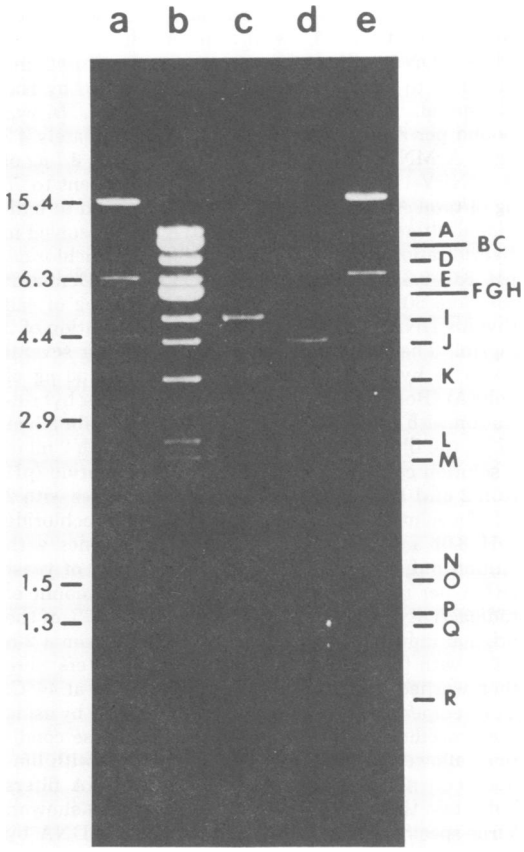

FIG. 1. *Electrophoresis of EcoRI-digested AcMNPV DNA (lane b) and purified EcoRI-I (lane c) and EcoRI-J (lane d) on 0.6% agarose gels. The AcMNPV DNA fragments are designated on the right as described previously (19). A HindIII digest of lambda DNA (lanes a and e) was included, and the molecular weights of the fragments (×10^6) are indicated on the left.*

Isolation of poly(A)$^+$ RNA. The precipitated RNA was dried in vacuo and then dissolved in high-salt buffer (10 mM Tris-hydrochloride, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.5% sodium dodecyl sulfate [SDS]) at a concentration of 2 mg/ml. The RNA was applied to an oligodeoxythymidylic acid cellulose column (volume, 0.4 ml), and the RNA lacking poly(A) was removed by washing the column extensively with high-salt buffer until no absorbance was detected. The poly(A)$^+$ RNA was eluted by washing the column with low-salt buffer (10 mM Tris-hydrochloride pH 7.5). The RNA was precipitated with ethanol at −20°C overnight, suspended in water, and stored at −70°C. About 60 μg of poly(A)$^+$ RNA was obtained from 1 mg of postmitochondrial RNA from infected cells.

Hybridization selection of viral RNA. Postmitochondrial RNA was hybridized to AcMNPV DNA

on nitrocellulose filters essentially as described by McGrogan et al. (15), with some modifications. AcMNPV DNA or DNA fragments were denatured and attached to nitrocellulose filters as described by Kafatos et al. (10). About 100 μg of AcMNPV DNA was bound per filter (diameter, 2 cm). Approximately 4.2 μg of AcMNPV DNA *Eco*RI fragment I and 3.7 μg of AcMNPV DNA *Eco*RI fragment J (equivalent to 75 μg of total AcMNPV DNA each) were bound to individual filters (diameter, 1 cm). RNA was dissolved in hybridization solution (120 mM Tris-hydrochloride, pH 8.0, 0.6 M NaCl, 4 mM EDTA, 2× Denhardt solution, 50% formamide, 0.25% SDS, 100 μg of calf thymus DNA per ml) at 37°C at a concentration of 2 mg/ml. The DNA filters were incubated for several hours in hybridization solution containing 50 μg of poly(A) (Boehringer Mannheim) per ml. After hybridization with 1 mg of postmitochondrial RNA for 8 h at 37°C, the filters were washed three times with 2 ml of a solution containing 10 mM Tris-hydrochloride (pH 8.0), 2 mM EDTA, and 0.5% SDS, three times with 2 ml of a solution containing 10 mM Tris-hydrochloride (pH 8.0) and 2 mM EDTA, and three times with elution buffer (2 mM EDTA, pH 7.0, 10 μg of yeast tRNA per ml) at room temperature. The amount of radioactivity in the final wash was about 0.07% of the original input (1.5×10^5 cpm/mg of RNA from a 2-h pulse with [^{3}H]uridine [see below]). The filters were then washed three times with elution buffer at 46°C. This temperature was determined empirically by using the procedure of McGrogan et al. (15). These conditions allowed us to remove RNA sequences with limited sequence homology from the viral DNA filters but retain the virus-specific hybrids (data not shown). Virus-specific RNA was removed from the DNA by washing the filters with elution buffer at 90°C for 3 min. The RNA was then precipitated with 2 volumes of ethanol in the presence of 0.2 M potassium acetate. About 35 μg of selected RNA was obtained from 1 mg of postmitochondrial RNA.

Labeling of RNA. The RNA in AcMNPV-infected cells was labeled in vivo with 100 μCi of [5,6-^{3}H]uridine (28 Ci/mmol; ICN) per ml of medium supplemented with 10% dialyzed fetal bovine calf serum from 19 to 21 h after the cells were infected. The ^{3}H-labeled RNA was used to monitor the RNA during the subsequent isolation procedures.

In vitro labeling of alkali-fragmented RNA was performed essentially by the method of Maizels (13). A 10-μg amount of poly(A)$^+$ of RNA was fragmented by treating it with 30 μl of 0.05 M Na_2CO_3 for 80 min at 50°C. After neutralization with an equal volume of 50 mM Tris-hydrochloride (pH 7.6)–0.05 M HCl and the addition of 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol, the RNA solution was stored at −20°C; 1 μg of fragmented RNA was mixed with 10 to 50 pmol of [γ-^{32}P]ATP (200 Ci/mmol; Amersham) and 1 U of T4 polynucleotide kinase (Boehringer Mannheim). After incubation for 30 min at 37°C, the reaction was stopped by adding 1 volume of 4% SDS. The labeled RNA was separated from the unincorporated radioactivity by gel filtration on Sephadex G-50, and the RNA solution was adjusted to 3× SSC (1× SSC is 0.15 NaCl plus 0.015 M sodium citrate).

RNA-DNA hybridization. After digestion with restriction endonucleases, the DNA fragments were electrophoresed on agarose gels and blotted onto nitrocellulose filters by the method of Southern (20). DNA blots were incubated overnight at 63°C in 3× SSC containing 100 μg of yeast RNA per ml. ^{32}P-labeled, alkali-fragmented poly(A)$^+$ RNA was then added, and the preparation was incubated for 40 h. The filters were then washed extensively with 3× SSC at 63°C, dried, and processed for autoradiography. Fast tungstate intensifying screens (Ilford) were used to reduce the exposure time.

In vivo labeling of proteins. Tritium-labeled AcMNPV extracellular virus and polyhedra were isolated from infected cells as described previously (18). To label cell proteins, IPLB-SF21 cells were infected at a multiplicity of 10 PFU/cell, washed twice at varying times after infection in defined medium (18) lacking L-leucine, and pulse-labeled for 3 h in the above-described medium containing 20 μCi of L-[4,5-^{3}H]leucine (46 Ci/mmol; ICN) per ml. At the end of each pulse, the labeling medium was removed, and the cells were washed twice with ice-cold phosphate-buffered saline and then boiled for 3 min in SDS protein disruption buffer (250 mM Tris-hydrochloride, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue).

In vitro protein synthesis. Translation of RNA was performed in an mRNA-dependent rabbit reticulocyte lysate cell-free protein-synthesizing system (16) that was obtained from Bethesda Research Laboratories, Inc., according to the directions of the manufacturer. A total of 5 μCi of L-[4,5-^{3}H]leucine (46 Ci/mmol; ICN) and 1 to 5 μl of RNA were added to the reaction mixture (final volume, 30 μl); 0.6 mM spermidine and 2 mM 2-mercaptoethanol were added to obtain optimal incorporation. Incubation was for 60 min at 30°C. The translation reaction was stopped either by adding 1 volume of SDS disruption buffer before electrophoresis or by adding 4 volumes of immunoprecipitation buffer (20 mM Tris-hydrochloride, pH 8.0, 0.4 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1.5% Nonidet P-40, 0.15% deoxycholate before immunoprecipitation.

Immunoprecipitation. Virus-specific proteins were precipitated from the cell-free translation mixture with serum prepared against either purified polyhedrin or occluded virus proteins of AcMNPV. The antisera were prepared essentially as described by Summers and Hoops (21). Cross-reactions between occluded virus proteins and serum prepared against purified polyhedrin did not occur, as determined by the new immunoautoradiographic techinque of Symington et al. (24), as modified by Smith and Summers (19a). A total of 10 μl of serum prepared against purified polyhedrin or occluded virus proteins was added to the translation mixture (150 μl) containing immunoprecipitation buffer. The immunoprecipitation reaction was allowed to proceed for 3 h at room temperature and then overnight at 4°C with occasional shaking. Then 30 μl of a 10% suspension of protein A-Sepharose CL-4B (Sigma Chemical Co.) in immunoprecipitation buffer was added, and the mixture was incubated for 60 min on ice with occasional shaking. The protein A-Sepharose-bound antigen-antibody complexes were washed twice with immunoprecipitation buffer by using differential centrifugation. The complexes were released from the protein A-

Sepharose by boiling in SDS disruption buffer for 3 min, and the protein A-Sepharose was removed by centrifugation. The supernatant was applied to a polyacrylamide gel.

SDS-polyacrylamide gel electrophoresis. Samples were analyzed on 10% polyacrylamide slab gels in 0.1% SDS by using the discontinuous Tris-glycine buffer system of Laemmli (11); electrophoresis was at a 9 W (constant power) for 2.5 to 3 h. Autoradiography was performed by the fluorographic method of Bonner and Laskey (4). The gels were exposed to Kodak X-Omat RP film at −80°C for varying times. ^{14}C-methylated myosin (molecular weight, 200,000), phosphorylase (100,000 and 92,000), bovine serum albumin (67,000), ovalbumin (46,000), and lysozyme (14,300) were obtained from Amersham and were included as molecular weight standards in the electrophoretic analyses. ^{14}C-methylated carbonic anhydrase (molecular weight, 30,000) was not used in molecular weight calculations because of its irregular mobility in polyacrylamide gels. The values for the molecular weights of the virus structural proteins and polyhedrin reported previously (18, 22) were revised (19a) after the separation methods were improved and new molecular weight standards were used.

RESULTS

Intracellular protein synthesis. The induction of ICSPS in AcMNPV-infected *S. frugiperda* cells was studied by analyzing proteins after 3-h pulses with [^{3}H]leucine at different times after infection (Fig. 2). We observed a gradual increase in the appearance of ICSPs during the infection. A number of these proteins had the same molecular weights as virion structural polypeptides (64,000, 55,000, 52,000, 41,000, 38,000, 33,000 and 7,500) and polyhedrin (33,000). Some ICSPs did not comigrate with virion structural polypeptides but were present in significant quantities in infected cells (molecular weights, 46,000, 39,000, 36,000, 27,000, 25,000, 20,000, 19,000, and 11,000). Host cell-specific protein synthesis gradually decreased after AcMNPV infection, and at later times the synthesis of virion structural polypeptides and ICSPs prevailed.

Although newly synthesized polyhedrin could be detected by a radioimmunoassay as early as 12 h p.i. (21), it was not until 16 h p.i. that polyhedrin was synthesized in significant quantities in infected cells (Fig. 2); this was concomitant with the appearance of polyhedra in infected cell nuclei. Radioactivity was incorporated into polyhedrin until at least 48 h p.i. In addition to polyhedrin, many ICSPs with molecular weights similar to those of virus structural polypeptides were detected at about 21 h p.i. Therefore, we arbitrarily decided to isolate cy-

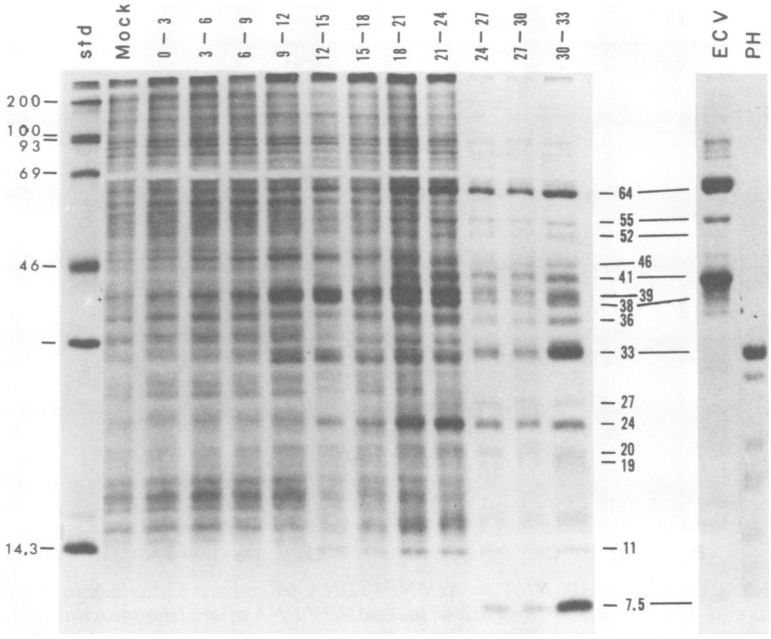

FIG. 2. *Autoradiogram of AcMNPV-infected S. frugiperda cells pulse-labeled with [^{3}H]leucine for 3 h at different times after infection. Extracellular virus (ECV) and polyhedrin (PH) were included for comparison. ^{14}C-methylated proteins were included as standards (std), and the molecular weights (×10^3) of these proteins are indicated on the left. The molecular weights (×10^3) of the ICSPs and the extracellular virus proteins are indicated on the right. The proteins were analyzed on a 10% polyacrylamide gel, and autoradiographic exposure was for 4 days at −80°C.*

toplasmic RNA at 21 h p.i. for our investigation of cytoplasmic transcripts and in vitro-synthesized proteins.

Characterization of poly(A)$^+$ RNA. We obtained poly(A)$^+$ RNA by oligodeoxythymidylic acid cellulose chromatography of postmitochondrial RNA from infected cells at 21 h p.i. This RNA was fragmented, end-labeled with ^{32}P, and hybridized to *Eco*RI and *Bam*HI digests of AcMNPV DNA, as described above. The [^{32}P]RNA hybridized to most, if not all, restriction fragments (Fig. 3), showing that at 21 h p.i. transcripts with sequences complementary to large portions of the genome were present. However, cytoplasmic poly(A)$^+$ RNA sequences from some fragments were more abundant than others, as judged by visual inspection of the autoradiograms. For example, relative to their genetic complexities, there were more (poly(A)$^+$ RNAs homologous to *Bam*HI-F (Fig. 3, lane d), *Eco*RI-I (lane c), *Eco*RI-P (lanes e and f), and double-digest fragments with molecular weights of 2.7×10^6, 2.2×10^6, 1.3×10^6 (*Eco*RI-P), and 1.2×10^6 (*Bam*HI-F) (lane e) than to other

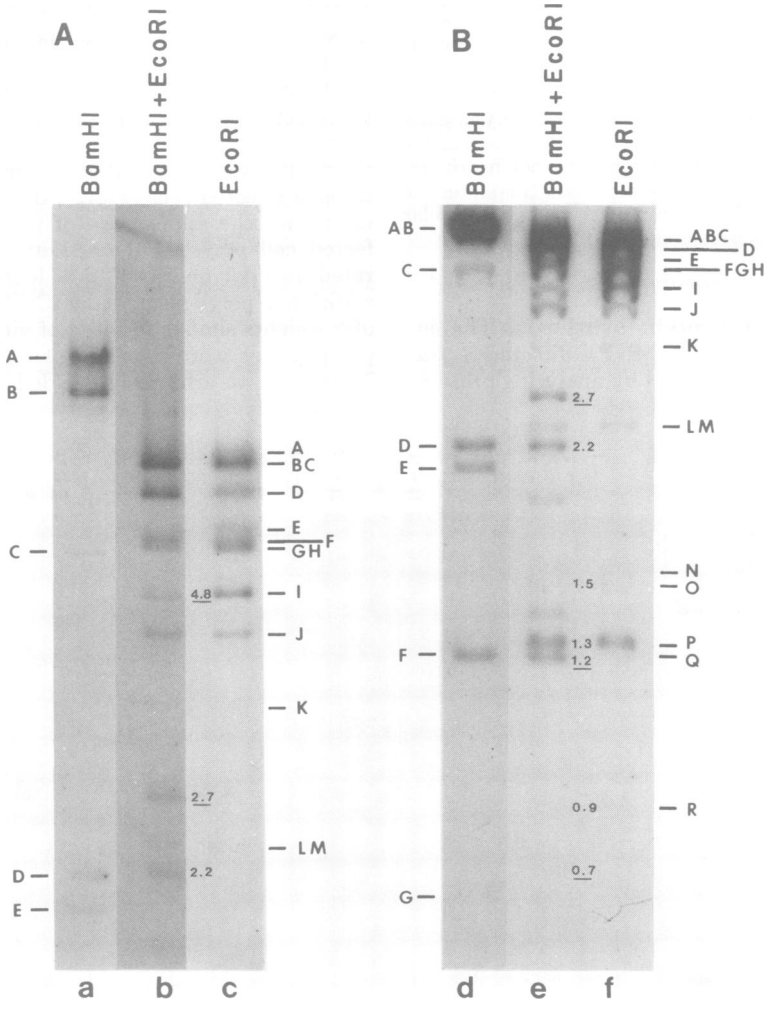

FIG. 3. *Hybridization of poly(A)$^+$ RNA to AcMNPV DNA fragments. Cytoplasmic poly(A)$^+$ RNA from infected cells was end-labeled with ^{32}P, hybridized to AcMNPV DNA after cleavage with BamHI (lanes a and d), EcoRI (lanes c and f), or both BamHI and EcoRI (lanes b and e), and transferred to nitrocellulose filters. Restriction digests were electrophoresed on 0.7% (A) and 1.2% (B) agarose gels. Hybridization with ^{32}P-labeled RNA (10^6 cpm/ml) was performed overnight at 63°C as described in the text. The designations of the EcoRI fragments (right) and the BamHI fragments (left) were according to Smith and Summers (18). The molecular weights ($\times 10^6$) of some the EcoRI-BamHI fragments (lanes b and e) are indicated on the gels; the fragments related to EcoRI fragment I underlined.*

fragments. Some fragments, such as *Bam*HI-C and *Eco*RI-A, -E, -K, and -N,O, did not hybridize to as many complementary poly(A)$^+$ RNA sequences.

In vitro translation of poly(A)$^+$ RNA and virus-specific RNA. Poly(A)$^+$ RNA and RNAs selected by hybridization to AcMNPV DNA from two separate preparations of RNA were translated in vitro, and the products were analyzed on polyacrylamide gels. The autoradiographic profiles obtained were virtually identical for poly(A)$^+$ RNA and virus-specific RNA (Fig. 4, lanes d and e). Another virus-specific RNA preparation (Fig. 4, lane c) produced essentially the same polypeptide pattern, suggesting that the hybridization selection procedure was reproducible. A large number of translation products were detected up to a molecular weight of 70,000. Polypeptides with molecular weights of 41,000, 39,000, 35,000, 33,000, 32,000, 31,000, 29,000, and 24,000 were synthesized in abundance, together with a number of low-molecular-weight polypeptides. In addition, a number of minor polypeptides (molecular weights, 64,000, 57,000, 54,000, 52,000, 50,000, 47,000, and 38,000) were also observed. Some polypeptides synthesized in vitro had electrophoretic mobilities similar to the mobilities of extracellular virus polypeptides (Fig. 4, lane f).

The 33,000-dalton polypeptide was identified as polyhedrin by comparing it with ^{3}H-labeled polyhedrin (Fig. 4, lane j) and with proteins immunoprecipitated with serum prepared against purified polyhedrin from in vitro-synthesized polypeptides (Fig. 4, lane i). The 41,000-dalton polypeptide had an electrophoretic mobility similar to that of a major AcMNPV nucleocapsid protein (Fig. 4, lane f). Moreover, this 41,000-dalton polypeptide could be immunoprecipitated from in vitro-synthesized polypeptides with serum prepared against occluded virus proteins (Fig. 4, lane g). After the same immuno-

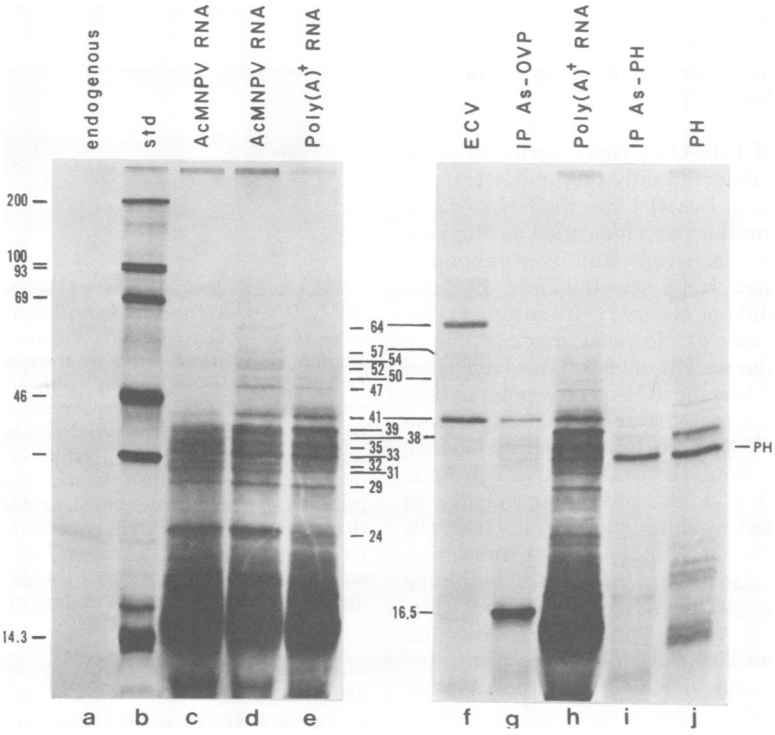

FIG. 4. *In vitro translation of poly(A)$^+$ RNA and virus-specific RNA. Poly(A)$^+$ RNA (lanes e and h) and RNA selected by hybridization to AcMNPV DNA (lanes c and d) were translated in a reticulocyte cell-free protein-synthesizing system, and the products were analyzed on 10% polyacrylamide gels as described in the text. Lane a contained products of endogenous protein synthesis. Translation products of virus-specific RNA were immunoprecipitated with antiserum against purified occluded virion proteins (IPAs-OVP; lane g) or polyhedrin (IPAs-PH; lane i). Partially purified polyhedrin (PH: lane j) and extracellular virus (ECV; lane f) were included as markers. ^{14}C-methylated protein standards (std) were included (lane b), and their molecular weights (×10^3) are indicated on the left. The molecular weights (×10^3) of the proteins in other lanes are indicated between the two panels. Autoradiographic exposure was for 22 days at −80°C.*

precipitation reaction we detected a number of other minor polypeptides, whose identities and relationships to the structural polypeptides of the virions have not been established yet. A protein with a molecular weight of 16,500 was also immunoprecipitated with serum prepared against occluded virus proteins (Fig. 4, lane g). This protein may have been another major in vitro translation product of both RNA preparations (Fig. 4, lanes c and d), but more proteins were present in this region of the gel. Among the less abundant translation products of the 21-h poly(A)$^+$ RNA was a polypeptide with a molecular weight of 64,000. A 64,000-dalton polypeptide was also detected in pulse-labeled AcMNPV-infected cells (Fig. 2), and this polypeptide had an electrophoretic mobility similar to that of another major virion protein.

In vitro translation of *Eco*RI-I- and *Eco*Ri-J-specific RNAs. Virus-specific RNAs were selected from *Eco*RI restriction fragments I and J and translated in vitro. *Eco*RI fragment I is located at map position 0 to 0.06, and *Eco*RI-J is located at map position 0.20 to 0.25 on the genome of AcMNPV (19) (By convention, the *Eco*RI restriction site between fragments I and B is used as the zero point on the circular DNA; the fragment order runs clockwise and is as follows: *Eco*RI-I,-R,-O,-A [manuscript in preparation].) We detected only one major translation product from *Eco*RI-I-specific RNA (Fig. 5, lane b). This product was identified as polyhedrin by its molecular weight and by immunoprecipitation. When RNA selected from *Eco*RI-J was used, we did not observe translation of polyhedrin (Fig. 5, lane d). However, another protein with a molecular weight of 39,000 was translated from *Eco*RI-J-specific RNA. This polypeptide, which was also a major translation product from total AcMNPV-specific RNA (Fig. 4, lane c), was probably a nonstructural AcMNPV protein. A protein with a similar molecular weight was also synthesized in abundance in infected cells at 21 h p.i. (Fig. 2). Minor in vitro translation products were also present on the gels containing both fragment-specific RNA preparations (Fig. 5, lanes b and d). A polypeptide with a molecular weight of about 30,000 seemed to be translated from *Eco*RI-I and -J RNAs. This polypeptide was not one of the endogenous translation products (Fig. 5, lane f) or one of the in vitro translation products from virus-specific RNA (Fig. 5, lanes a and e).

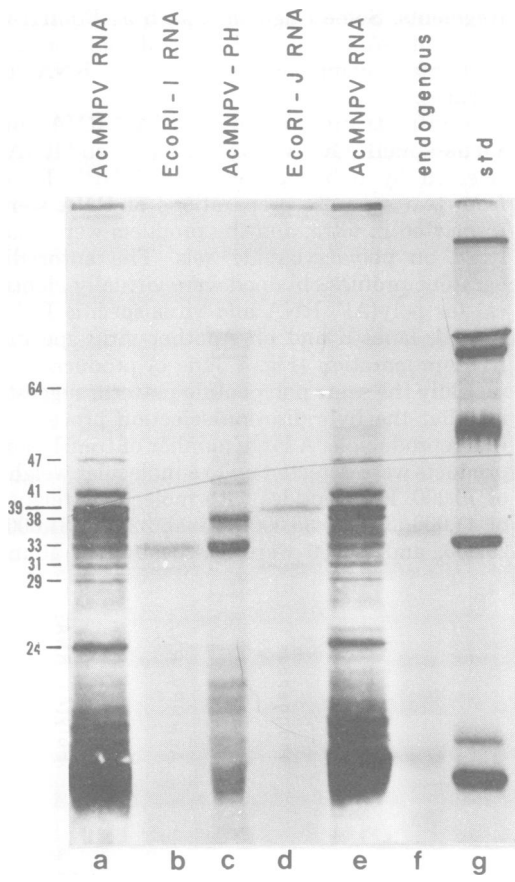

FIG. 5. *In vitro translation of fragment-selected RNA. RNAs selected by hybridization to AcMNPV DNA (lanes a and e), EcoRI fragment I (lane b), and EcoRI fragment J (lane d) were translated in a reticulocyte cell-free protein-synthesizing system and analyzed on a 10% polyacrylamide gel as described in the text. Partially purified polyhedra (PH) were analyzed to indicate the position of the polyhedrin (lane c). Lane f contained the endogenous translation products of the cell-free system, and lane g contained the ^{14}C-methylated protein standards (std). The molecular weights (×10^3) of some of the in vitro-synthesized products are indicated on the left. Autoradiographic exposure was for 22 days at −80°C.*

DISCUSSION

The appearance of ICSPs at different times after infection suggested a temporal regulation of AcMNPV-directed protein synthesis. Detailed information regarding the appearance and disappearance of these ICSPs has been obtained, and several transitional phases in the replicative cycle of AcMNPV have been recognized and proposed (5, 8, 14, 32). Some ICSPs, such as polyhedrin (molecular weight, 33,000) and two major enveloped nucleocapsid proteins (molecular weights, 64,000 and 41,000), are synthesized predominantly late after infection. In this study we isolated poly(A)$^+$ RNA and virus-specific RNA from AcMNPV-infected cells at about 21

h p.i., a time when most of the mRNA sequences for viral structural proteins were probably present in infected cells.

Using the blot hybridization technique, we detected the sequence homologies of poly(A)$^+$ RNA with fragments representing the whole AcMNPV genome. However, the ^{32}P-labeled RNA was not distributed evenly among the different DNA fragments. This showed that at 21 h p.i. poly(A)$^+$ RNA was present in more abundance from some regions of the genome than from others. The difference in the relative concentrations of poly(A)$^+$ RNAs may reflect controls at the level of primary transcription, processing, and transport and the stabilities of the different RNAs in the cytoplasm. Also, although the poly(A)$^+$ RNA was isolated late after infection, it is possible that RNA made earlier after infection continued to be present at later times.

The polypeptides synthesized in vitro from poly(A)$^+$ RNA and RNA selected from AcMNPV DNA (virus-specific RNA) were virtually identical both in number and in quantity. It might be assumed that the size distributions of the two preparations of RNA were also very similar, as described previously by Van der Beek (28) for a similar AcMNPV RNA preparation. An analysis of labeled virus-specific RNAs by oligo-deoxythymidylic acid cellulose chromatography showed that at least 50% of the RNA was retained by the column (data not shown). Therefore, we concluded that the poly(A)$^+$ RNA in AcMNPV-infected cells at 21 h p.i. contained primarily virus-specific RNA. This suggests that mechanisms of post-transcriptional polyadenylation operate in baculovirus transcription, as is the case in most other animal and plant virus systems. The mRNA for polyhedrin is apparently also polyadenylated. The presence of at least one abundant mRNA with a molecular weight of approximately 240,000, which is sufficient to code for polyhedrin, has been described previously (27).

A number of distinct proteins were synthesized in vitro from virus-specific RNA. The identity of the 41,000-dalton polypeptide as one of the major nucleocapsid proteins and the identity of the 33,000-dalton polypeptide as polyhedrin were established by the electrophoretic mobilities of these polypeptides and by immunoprecipitation. These results support previous conclusions from recombination studies that both proteins are virus coded (23). The similar electrophoretic mobilities of the in vivo- and in vitro-synthesized polyhedrins suggest that in infected cells considerable post-translational modification of this protein may not occur. Extensive glycosylation, methylation, and phosphorylation of polyhedrin have not been observed in vivo (8, 14). Furthermore, post-transcriptional cleavage of a larger precursor polypeptide is unlikely, as the in vivo- and in vitro-synthesized polyhedrins have the same molecular weight. Precursors of polyhedrin in infected cells have been reported (5), but this observation was not confirmed by other workers (8, 14, 32). The relationship between the other in vitro products and the structural proteins of the virion or nonstructural ICSPs is conjectural, since identifications of proteins synthesized in vitro and in infected cells cannot be made by comparing electrophoretic mobilities alone. However, it is possible that the 39,000- and 35,000-dalton in vitro-synthesized polypeptides could be identical to the two major ICSPs with these molecular weights synthesized in infected cells.

By hybridization selection of viral RNA to DNA fragments instead of intact AcMNPV DNA and in vitro translation of these RNAs, we demonstrated that the mRNA sequences directing the synthesis of polyhedrin are located on *Eco*RI fragment I of the AcMNPV genome. An analysis of intertypic recombinants between *R. ou* and AcMNPV indicated previously that *Eco*RI-I may contain the genetic information for polyhedrin (23). Our experiments with *Eco*RI-I-selected RNA support the hypothesis that the structural gene for polyhedrin is located on *Eco*RI-I. The coding sequence for polyhedrin (molecular weight, 33,000) occupies a minimum of about 0.7×10^6 daltons of DNA, which would leave enough room for at least several other genes on *Eco*RI-I (molecular weight, 4.8×10^6). When we used RNA specific for *Eco*RI-J, polyhedrin was not made; this demonstrated the specificity of the hybridization selection procedure. However, a polypeptide with a molecular weight of 39,000 was translated in vitro from *Eco*RI-J-specific RNA. This protein might be identical to the major 39,000-dalton ICSP in infected cells. A polypeptide with a molecular weight of about 30,000 seemed to be translated from both *Eco*RI-I and *Eco*RI-J. Since this protein was not detected among the in vitro translation products of virus-specific RNA, it may have been either a minor virus-encoded product or an endogenous protein whose synthesis was enhanced (6).

However, it is possible that leader or promoter sequences are responsible for the hybridization of mRNA with *Eco*RI-I and -J. The generation of mRNA's by splicing mechanisms has been reported previously in several animal-virus systems examined (1, 2, 30), but in not all such systems (31). It is not known whether this mechanism of post-transcriptional modification op-

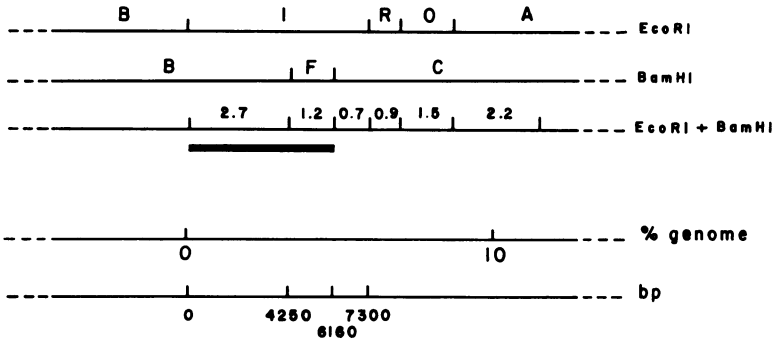

FIG. 6. *Restriction map of AcMNPV DNA between positions 0 and 0.10. The cleavage sites of EcoRI and BamHI and the molecular weights of the double-digest fragments (×10⁶) are indicated according to Smith and Summers (19). The heavy bar represents the section of the DNA segment which showed relatively increased hybridization with cytoplasmic transcripts. bp, Base pairs.*

erates in baculovirus replication. Experiments involving hybrid-arrested translation (17), R-looping (25), and S1 digestion of RNA-DNA hybrids (3) eventually will lead to an answer to this question.

A more precise location of the polyhedrin gene in fragment *Eco*RI-I was suggested by the results of our Southern hybridization experiments. Poly(A)$^+$ RNA hybridized slightly more to *Bam*HI fragment F and a double-digest fragment of 2.7×10^6 daltons and considerably less to *Bam*HI fragment C and a double-digest fragment of 0.7×10^6 daltons (Fig. 3, lane e). *Eco*RI fragment I was separated by restriction endonuclease *Bam*HI into three fragments with molecular weights of 2.7×10^6, 1.2×10^6, and 0.7×10^6 (19) (Fig. 6), and the 1.2×10^6-dalton fragment corresponded to *Bam*HI fragment F. With this information, we speculated that the polyhedrin gene is located near the middle or left end of *Eco*RI fragment I, between map positions 0 and 0.045 (Fig. 6). Experiments similar to those described here but with smaller restriction fragments from within *Eco*RI-I will be performed to test this hypothesis.

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