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Expression of Aleutian Mink Disease Parvovirus Proteins in a Baculovirus Vector System

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We have previously published a detailed transcription map of Aleutian mink disease parvovirus (ADV) and proposed a model for the translation of the two virion structural proteins (VP1 and VP2) and three nonstructural proteins (NS-1, NS-2, and NS-3) (S. Alexandersen, M. E. Bloom, and S. Perryman, J. Virol. 62:3684–3994, 1988). To verify and further characterize this model, we cloned the predicted open reading frames for NS-1, NS-2, NS-3, VP1-VP2, and VP2 alone into a recombinant baculovirus and expressed them in Sf9 insect cells. Expression of VP1-VP2 or VP2 alone in cDNA and in the genomic form was achieved. The expressed proteins had molecular weights similar to those of the corresponding proteins of wild-type ADV-G, although the ratio of VP1 to VP2 was altered. The recombinant baculovirus-expressed ADV VP1 and VP2 showed nuclear localization in Sf9 cells and were able to form particles indistinguishable, by electron microscopy, from wild-type virus. The large nonstructural protein, NS-1, showed predominantly nuclear localization in Sf9 cells when analyzed by immunofluorescence and had a molecular weight similar to that of wild-type ADV NS-1. Moreover, expression of NS-1 in Sf9 cells caused a change in morphology of the cells and resulted in 10-times-lower titers of recombinant baculovirus during infection, suggesting a cytostatic or cytotoxic action of this protein. The smaller NS-2 gene product seems to be located in the cytoplasm. When analyzed by Western immunoblotting, NS-2 comigrated with an approximately 16-kDa band seen in lysates of ADV-infected feline kidney cells. The putative NS-3 gene product exhibited a diffuse distribution in Sf9 cells and had a molecular weight of approximately 10,000. All of the expressed ADV-encoded proteins were recognized by sera from ADV-infected mink. Thus, expression of ADV cDNAs allowed assignment of the different mRNAs to the viral proteins observed during ADV infection in cell culture and supported our previously proposed ADV transcriptional and translational scheme. Moreover, the production of structural proteins from a full-length NS-2 mRNA may add to the repertoire of parvovirus gene expression.

Aleutian mink disease parvovirus (ADV) is an autonomously replicating parvovirus which causes a number of interesting clinical and pathological syndromes in mink. These syndromes range from acute pneumonia in neonatal mink kits (3, 4, 7, 8) to development of a low-level persistent infection in adult mink manifested by hypergammaglobulinemia, chronic immune complex-mediated glomerulonephritis, and arteritis (6, 12, 15, 32, 51, 52).

ADV encapsidates a linear single-stranded DNA genome with a size of approximately 4,800 nucleotides. The 5′ and 3′ termini contain different palindromic sequences necessary for replication (13, 14). Viral transcription is regulated by cellular and viral products and takes place from two promoters located in the left part and in the middle of the genome (5, 14). The transcription program uses overlapping transcripts and multiple reading frames to increase the genetic information included in the small genome (5, 14).

We have previously published a detailed transcriptional map and proposed a translational scheme for ADV based on Northern (RNA) blotting, cDNA cloning, and primer extension analysis (5). The structures of five mRNAs designated R1 (4.3 kb), R2 (2.8 kb), R2′ (0.85 kb), R3 (2.8 kb), and RX (1.1 kb) were proposed (5). Three putative nonstructural proteins, NS-1, NS-2 and NS-3, were tentatively assigned to be encoded by four spliced RNAs (R1, R2′, R2, and RX) transcribed from a promoter at map unit 3. Protein analysis had defined the expression of the NS-1 protein and indicated the presence of NS-2 during ADV infection in vivo and in cell culture (8, 16, 53), while no data for the putative NS-3 protein exist.

Transcripts from the left side of the genome are known from other parvoviruses to code for a group of virus Rep or nonstructural proteins necessary for replication (11, 26). The large NS-1 protein is a nuclear protein which has several intrinsic enzymatic properties. It shows site-specific nickase, helicase, ATPase, and DNA binding activities (35, 65) and is also involved in regulation of the viral and cellular promoters (2, 30, 46, 54, 55, 62). Another feature of the parvovirus NS-1 protein is a cytotoxic or cytostatic action on the host cell (43, 48).

While the NS-1 proteins of most parvoviruses are fairly well characterized, not much is known about the other nonstructural proteins. For minute virus of mice (MVM), NS-2 has been described as consisting of phosphorylated isoforms distributed in the cytoplasm and nucleus of the cell (27). The NS-2 gene seems to be necessary, in a cell-type-specific manner, for efficient genome replication and virus production (44, 47).

The virion of ADV consists of two different polypeptides with overlapping amino acid sequences (5, 14). It has been suggested that these structural proteins (VP1 and VP2) are generated by translation initiation at two different ATGs within a single spliced RNA transcript (R3) starting from a

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promoter around map unit 36 (5, 14). In the other parvoviruses, the different structural proteins are normally translated from alternative spliced mRNAs and sometimes further modified by posttranslational proteolytic processing after assembly into virions (26, 37, 40, 61).

Because of the multifunctional properties of the virally encoded proteins, characterization of ADV gene products might yield important information about the pathogenesis of ADV infection in mink. Therefore, to characterize the gene products and assign them to the viral proteins seen during ADV infection, the open reading frames (ORFs) proposed from the ADV transcription program were constructed from different cDNAs and genomic DNA and expressed in insect cells by using a baculovirus vector. The results supported the proposed transcriptional and translational model of ADV and revealed interesting features of translation of ADV proteins.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* (Sf9) insect cells and the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) were obtained from InVitrogen (San Diego, Calif.). Cells were cultured in Grace medium supplemented with 10% fetal calf serum (FCS), yeastolate, and 50 μg of gentamicin per ml in either tissue culture flasks or spinner bottles at 28°C by the procedures described by Summers and Smith (60). Crandell feline kidney (CRFK) cells (28) were cultured in Dulbecco minimal essential medium supplemented with 5% FCS at 32°C in a 5% CO_2 humidified atmosphere. The cell culture-adapted ADV-G strain was kindly provided by Ebba Lund, The Royal Veterinary and Agricultural University of Copenhagen, Frederiksberg, Denmark. Propagation of ADV-G was performed as described before (7).

Bacteria. Two *Escherichia coli* strains, JM109 (Promega Corp., Madison, Wis.) and Sure (Stratagene, La Jolla, Calif.), were transformed as described by Maniatis et al. (45).

Antiserum. The antiserum was a pool of sera from ADV-infected mink. Fluorescein isothiocyanate (FITC)-conjugated protein A was obtained from Dako A/S (Glostrup, Denmark). Purification of mink immunoglobulin G and FITC conjugation were performed as previously described (23).

Cytospin preparations and immunofluorescence. Cytospin preparations were made from 2×10^4 Sf9 cells infected with recombinant AcNPV viruses. After being air dried, the cells were fixed for 4 min in acetone at −20°C. The slides were incubated in phosphate-buffered saline (PBS) containing 2% FCS and 5 μg of FITC-conjugated ADV-positive mink immunoglobulin G or a 1/100 dilution of ADV antiserum for 1 h at room temperature. The slides were washed three times in PBS and, for the indirect technique, incubated for 1 h with FITC-protein A at a concentration of 5 μg/ml. After being mounted in DABCO [1,4-diazabicyclo(2,2,2)octane] reagent (250 mg in a mixture of 9 ml of glycerol and 1 ml of PBS [pH 8.6]), the cytospin preparations were examined by fluorescence microscopy.

Chemicals and reagents. All enzymes were obtained from Promega, Boehringer (Mannheim, Germany), InVitrogen, or United States Biochemical (Cleveland, Ohio). Plasmids were purchased from either Promega (pGEM3Z) or InVitrogen (pBlueBac βII). [^{35}S]dATP and ^{125}I-protein A were obtained from Du Pont, New England Nuclear Corp. (Boston, Mass.). Synthetic oligonucleotides specific for plasmid DNA were from Promega. ADV-specific oligonucleotides were synthesized as described previously (14). Cell culture equipment was obtained from Nunc (Roskilde, Denmark). Tunicamycin was from Sigma Chemical Company (St. Louis, Mo.), and Bluo-Gal was from GIBCO/BRL (Grand Island, N.Y.).

Construction of recombinant AcNPVs. Recombinant AcNPVs were constructed with the transfer vector pBlueBac βII (64), which contains an *Nhe*I site approximately 50 nucleotides from the polyhedrin mRNA start site. This vector also contains a β-galactosidase gene driven by the AcNPV ETL promoter.

Construction of the NS-1 gene. When we originally did cDNA cloning from ADV-infected cell cultures, no full-length NS-1 cDNAs were obtained (5). Therefore, we constructed the putative ORF by cloning a combination of fragments from a genomic clone (pADVG IQ-6 [14], kindly provided by Marshall Bloom, Rocky Mountain Laboratories, Hamilton, Mont.) and two cDNA clones (3B2 and 25.31) described previously (5) (Fig. 1). An *Alw*NI-*Xho*I fragment (nucleotides 242 to 2058) from pADVG IQ-6 was used to replace the corresponding fragment from clone 3B2; the ADV B splice (5) was inserted into this clone with an *Nco*I-*Xho*I fragment from clone 25.31 (5). This procedure yielded a clone consisting of ADV nucleotides 196 to 1961, 2042 to 2213, and 2287 to 2353 flanked by *Xba*I linkers.

The NS-2, NS-2Long, and NS-3 genes were full-length cDNA clones consisting of ADV nucleotides 196 to 384, 2042 to 2213, and 2287 to 2353 (NS-2), 198 to 384, 2042 to 2213, and 2287 to 4424 (NS-2Long), and 196 to 384, 1737 to 1818, 2042 to 2213, and 2287 to 2353 (NS-3), all flanked by *Xba*I linkers.

Expression of VP1-VP2 and VP2 alone was achieved by cloning an *Xho*I-*Xba*I fragment (nucleotides 2058 to 2213 and 2287 to 4424) and an *Hgi*AI-*Xba*I fragment (nucleotides 2317 to 4424) from clone IB21 (5), respectively. The putative start ATGs for VP1 and VP2 are positioned at nucleotides 2204 and 2406. To express VP1 and VP2 from an unspliced ADV genomic form, an *Xho*I-*Bam*HI fragment from pADVG IQ-6 (nucleotides 2058 to 4592) was blunt ended with Klenow enzyme, and then *Xba*I linkers were ligated to the fragment. Since *Xba*I is compatible with *Nhe*I, all of the constructed genes could be cloned into the AcNPV transfer vector pBlueBac βII.

These procedures generated plasmids pBlueBac-NS-1, pBlueBac-NS-2, pBlueBac-NS-2Long, pBlueBac-NS-3, pBlueBac-VP1,2(cDNA), pBlueBac-VP2(cDNA), and pBlueBac-VP1,2(genomic DNA). The structures of cloned genes were confirmed by dideoxy sequencing as previously described (5, 14).

DNA transfection. Plasmids containing the foreign genes were transfected into Sf9 cells with Lipofectin (GIBCO/BRL). One microgram of wild-type AcNPV DNA and 2 to 4 μg of recombinant transfer vector in 50 μl of distilled H_2O were mixed with 30 μg of Lipofectin in a total of 50 μl of distilled H_2O. After 15 min of incubation at room temperature, the mixture was added dropwise to 2×10^6 Sf9 cells (in a 25-cm^2 tissue culture flask) in 3 ml of serum-free Grace medium and incubated for 6 h. At that time, the medium was changed to normal fresh medium and incubated for 3 to 5 days.

Purification of recombinant AcNPV. The supernatant from the transfected Sf9 cells was plaque purified once essentially as described previously (60). The blue plaques were picked, and virus was eluted overnight in 1 ml of insect medium. The eluted virus was then diluted 10^3-, 10^4-, and 10^5-fold and distributed in 96-well microtiter plates containing 10^4 Sf9 cells per well (48 wells for each dilution step). After 5 to 7

days, 30 μg of Bluo-Gal was added to the wells and incubated for 1 to 2 days. Blue wells at the highest virus dilutions were then screened by making cytospin preparations of the cells and then performing immunofluorescence assays with a FITC-conjugated polyclonal antibody against ADV proteins. Normally, recombinant virus, isolated from wild-type virus, was obtained in this step. In a few cases, the procedure had to be repeated to get recombinant AcNPV free of wild-type AcNPV.

PAGE and immunoblots. Total cellular proteins were lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM EDTA, 0.15 M NaCl, 20 mM Tris-HCl [pH 7.4], 1 mM phenylmethylsulfonyl fluoride, 0.5 U of aprotinin per ml, 5 μg of leupeptin per ml). Before electrophoresis, the lysates were boiled in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and sonicated. Samples were subjected to electrophoresis in 10 or 15% denaturing acrylamide gels by the procedure of Laemmli (41). Proteins were stained with Coomassie blue or blotted onto nitrocellulose membranes by using a semidry blotting system according to the manufacturer's instructions (JKA-BIOTECH, Brønshøj, Denmark). The membranes were blocked for 2 h in a mixture of 10% nonfat milk and 0.05% Tween 20 in PBS at 4°C and incubated with a 100-fold dilution of antibody in this solution overnight. After three washes in PBS containing 0.05% Tween 20 at 4°C, the membranes were incubated for 2 h with 2 μCi of ^{125}I-protein A in 20 ml of washing buffer. After six washes, the membranes were subjected to autoradiography. Exposure times were between 2 and 20 h.

Purification of ADV-like particles. AcNPV-infected Sf9 cells in PBS were sonicated three times for 15 s each time, and cellular debris was removed by centrifugation (5,000 × *g* for 20 min at 4°C). The supernatant was either layered on top of a solution of 40% (wt/vol) sucrose in PBS and centrifuged at 150,000 × *g* for 4 h or layered on top of 12 ml of 39% (wt/vol) cesium chloride solution and centrifuged at 240,000 × *g* with an SW41 rotor for 24 h at 19°C. Fractions were analyzed for ADV antigens by countercurrent electrophoresis (22). The densities of the fractions were determined by refractometry.

Electron microscopy. Particles purified by sucrose centrifugation were absorbed on Formvar carbon-coated grids negatively stained with 2% uranyl acetate and examined with a JEOL JM100B electron microscope as previously described (3).

RESULTS

Expression of recombinant ADV VP1 and VP2 proteins. To examine the expression of ADV VP1 and VP2, three different recombinant AcNPVs were purified. Two recombinant AcNPVs potentially coding for VP1-VP2 [VP1&2(cDNA)$_{AC}$] and VP2 [VP2(cDNA)$_{AC}$] were generated from cDNA forms of the genes. VP1&2(cDNA)$_{AC}$ contained the overlapping ORFs for VP1 and VP2 and the expected ATGs for initiation of both proteins (Fig. 1). VP2(cDNA)$_{AC}$ had the putative ORF for VP2 only. A recombinant AcNPV containing the genomic segment of VP1&2 [VP1&2(DNA)$_{AC}$], was also constructed. VP1&2(DNA)$_{AC}$ had a nucleotide sequence in the coding region identical to that of VP1&2(cDNA)$_{AC}$ except that the spliced-out intron sequence at nucleotides 2214 to 2286 (splice C [5]) was present in the former gene construct. SDS-PAGE and Western blot (immunoblot) analysis of total lysates of monolayers of Sf9 cells infected with VP1&2(cDNA)$_{AC}$, VP2(cDNA)$_{AC}$, and VP1&2(DNA)$_{AC}$ at high multiplicities (~10) harvested 48 h postinfection (p.i.) showed high levels of expression of ADV structural proteins (Fig. 2A and B, lanes 1 to 3). The recombinant AcNPVs VP1&2(cDNA)$_{AC}$ and VP2(cDNA)$_{AC}$ expressed polypeptides VP1&2 and VP2 alone, respectively. The observed mobilities in SDS-PAGE were similar to those of the virus structural proteins p85 and p75 (15, 16), which are expressed during ADV-G infection in CRFK cells. The ratio of VP1 to VP2 when these proteins are expressed by VP1&2(cDNA)$_{AC}$ is reversed in comparison with the VP1/VP2 ratio reported for ADV-G (16). Analysis of the Coomassie blue-stained gel by laser densitometry suggested that between 10 and 20% of the total protein in the lysates was recombinant protein, corresponding to 20 to 40 mg/10^9 Sf9 cells (17). Infection of Sf9 cells with VP1&2(DNA)$_{AC}$ revealed only expression of VP2 (Fig. 2A and B, lanes 3), supporting the observation that splicing of AcNPV-expressed RNA is ineffective during infection (36, 42). Also, this construct had a lower level of expression than the cDNA form of the gene. Propagation of the viruses in Sf9 cells in the presence of the N-linked glycosylation inhibitor tunicamycin had no effect on the mobility of the expressed proteins in Sf9 cells, indicating that these proteins, as in normal ADV-G infection, are not modified by glycosylation (data not shown). Immunofluorescence analysis of Sf9 cells infected with VP1&2(cDNA)$_{AC}$ and VP2(cDNA)$_{AC}$ (Fig. 3A and B) showed that VP1 and VP2 were localized in the nucleus of the cells. This finding suggests that both proteins can be transported independently into the nucleus, probably by using a common nuclear localization signal located in their overlapping amino acid sequence.

Formation of parvovirus-like particles by recombinant AcNPV. To determine whether recombinant AcNPV VP1&2 (cDNA)$_{AC}$ and VP2(cDNA)$_{AC}$ could synthesize particles, we infected Sf9 cells at high multiplicities. At 48 h p.i., the cells were harvested, sonicated, and analyzed by electron microscopy after ultracentrifugation through a sucrose cushion. Both VP1&2(cDNA)$_{AC}$ and VP2(cDNA)$_{AC}$ formed icosahedral particles of approximately 25 nm in size (Fig. 4) which banded in cesium chloride gradients at a density of 1.33 g/ml. This is in agreement with the density of empty particles generated during ADV-G infection in CRFK cells (15, 21). To investigate whether the assembled particles expressed by VP1&2(cDNA)$_{AC}$ had the same VP1/VP2 ratio as that observed in the total lysates (10:1), the particles were analyzed by SDS-PAGE after purification through a sucrose cushion (Fig. 5, lane 1). A VP1/VP2 ratio similar to that in total lysates was observed, suggesting that particle formation in the AcNPV expression system is independent of the ratio of VP1 to VP2. Moreover, particles consisting of VP2 only or of a majority of VP1 can be formed. The antigenicities of VP1 and VP2 were tested against a panel of sera from ADV-infected mink by Western blotting and countercurrent electrophoresis (22). No difference between the antigenicities of the AcNPV products and ADV-G could be detected in these assays (data not shown). The immunogenicity of purified ADV-like particles generated from VP1&2 (cDNA)$_{AC}$ also seems to be promising because the particles were able to induce a considerable antibody response against ADV structural proteins in immunized mink (1).

Expression of ADV proteins from recombinant AcNPVs harboring the NS-1, NS-2, NS-2Long, and NS-3 genes. To examine the proteins expressed by the putative mRNAs for the ADV NS proteins, the proposed ORFs for these proteins (Fig. 1) were inserted downstream of the polyhedrin promoter in recombinant AcNPVs and expressed after infection in Sf9 cells. These recombinant AcNPVs are designated

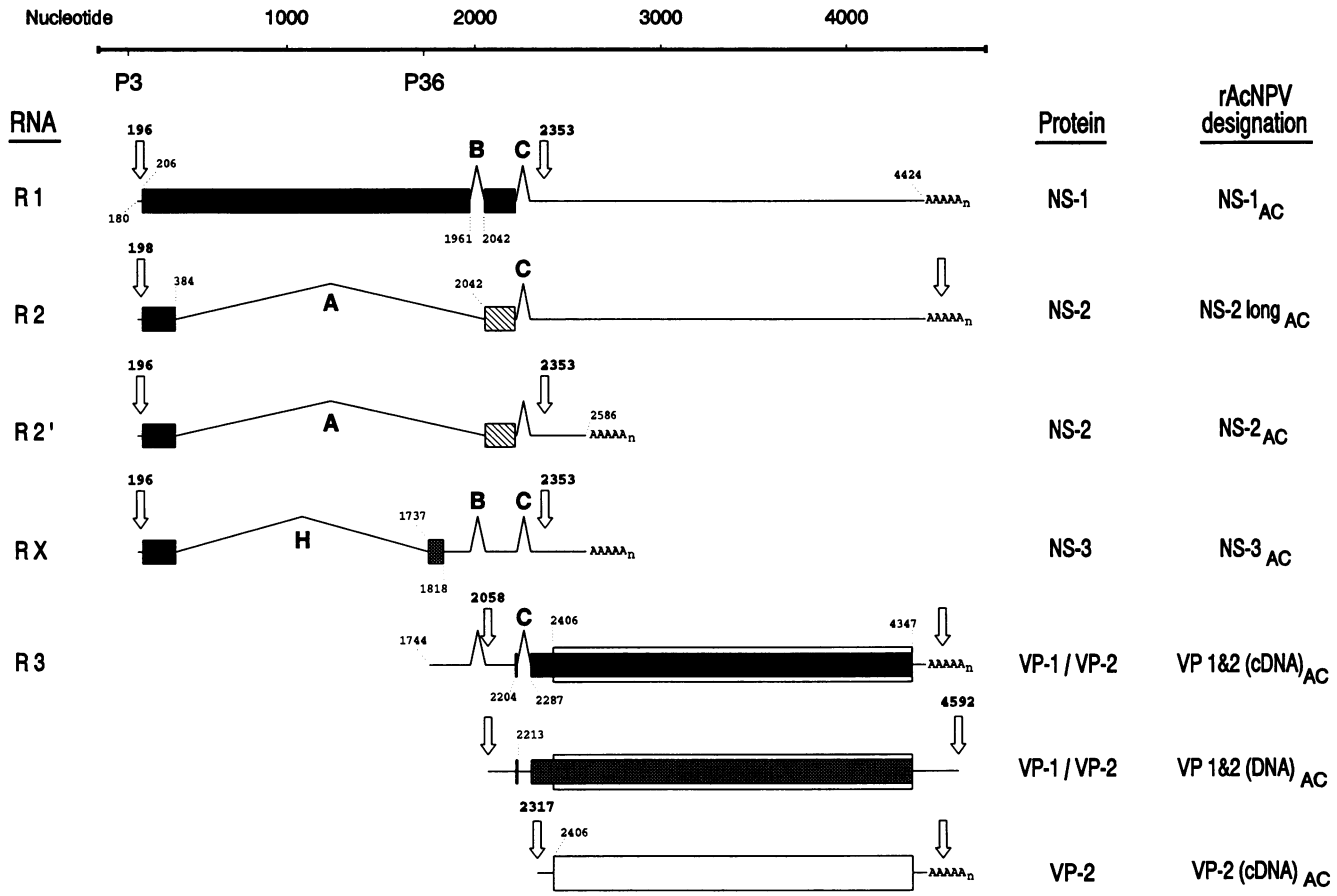

FIG. 1. Diagram of the cloned ADV genes expressed by recombinant AcNPV (rAcNPV). The ADV sequences located between the arrows (indicated in nucleotides) were cloned into a baculovirus transfer vector and inserted into the AcNPV genome by recombination. The upper portion of the figure depicts ADV nucleotide numbers and positions of the P3 and P36 promoters. The nomenclature and the predicted structure of the ADV mRNAs are shown with the 5′ end at the left. Horizontal lines indicate RNA sequences, peaks indicate spliced introns, and boxes indicate ORFs presumably used in translation of the RNAs. At the right are the putative proteins expressed by the corresponding ADV genes and the designations of the recombinant baculoviruses harboring these genes. The R3 mRNA encodes both VP-1 and VP-2. VP-1 and VP-2 have overlapping amino acid sequences, and their ORFs are depicted by a black box and a white box, respectively.

$NS\text{-}1_{AC}$, $NS\text{-}2_{AC}$, $NS\text{-}2Long_{AC}$, and $NS\text{-}3_{AC}$. Analysis of total lysates of Sf9 cells infected with $NS\text{-}1_{AC}$, $NS\text{-}2_{AC}$, and $NS\text{-}3_{AC}$ by SDS-PAGE 48 h p.i. showed high levels of expression of ADV proteins (Fig. 6A and B, lanes 1 to 3). Western blot analysis of similar lysates, using sera from infected mink as the antibody source, also clearly identified the expressed NS proteins. $NS\text{-}1_{AC}$ expressed a protein (Fig. 6B and C) which comigrated with the putative NS-1, formerly designated p71 (16), and $NS\text{-}2_{AC}$ expressed a protein that comigrated with NS-2 in total lysates from ADV-G-infected CRFK cells. The minor bands in $NS\text{-}1_{AC}$ (Fig. 6B and C) are presumably proteolytic degradation products, since the intensity of these bands could be reduced by inclusion of a cocktail of protease inhibitors (Fig. 6C, lane 3). The NS-2 gene product had a molecular weight of about 16,000, which is somewhat higher than the molecular weight calculated on the basis of the sequence (13,400) and the molecular weight that has been reported previously (5, 53). The observed slower mobility may be due to posttranslational modifications. The NS proteins are known to be phosphoproteins (25, 27), and phosphorylation could give this group of proteins a slower mobility in gels. High expression of recombinant protein from $NS\text{-}3_{AC}$ was also achieved. NS-3 migrated with a molecular weight of about 10,000 in SDS-PAGE, in agreement with the size estimated from the sequence (5). However, no bands of that size could be identified in lysates from ADV-G-infected CRFK cells. This could at least in part be explained by the low abundance of this mRNA in ADV-G-infected CRFK cells (5). Immunofluorescence analysis of Sf9 cells infected with $NS\text{-}1_{AC}$ showed that NS-1 is localized in the nucleus of the cells (Fig. 3C). Similar nuclear localization could be observed in CRFK cells and COS-7 cells transfected with the NS-1 gene in a eukaryotic expression vector (data not shown). Sf9 cells infected with $NS\text{-}1_{AC}$ had a morphology different from that of Sf9 cells infected with $NS\text{-}2_{AC}$ or $NS\text{-}3_{AC}$. While the size of the nuclei in $NS\text{-}2_{AC}$- or $NS\text{-}3_{AC}$-infected cells seemed to increase during infection, the nuclei in the $NS\text{-}1_{AC}$-infected cells slowly decreased in size (data not shown). Furthermore, during amplification of $NS\text{-}1_{AC}$, the obtained virus titer was approximately 10 times lower than the observed titer for the other recombinant AcNPV. This might be explained by the cytotoxic or cytostatic action of this protein, as reported for NS-1 expressed by parvoviruses such as adeno-associated virus, MVM, human parvovirus B19, and rodent parvovirus H-1 (25, 33, 43, 48). In this

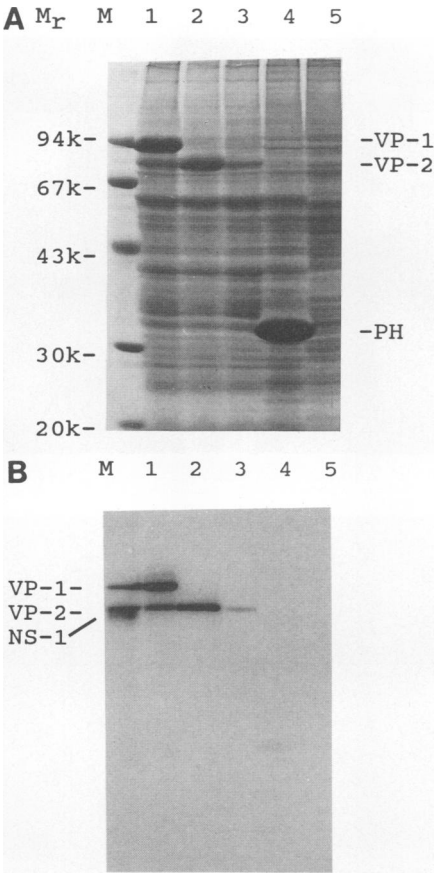

FIG. 2. Coomassie blue-stained SDS–10% polyacrylamide gel (A) and immunoblot (B) analysis of total lysates of Sf9 cells infected with three different recombinant AcNPVs expressing the structural proteins of ADV. The Sf9 cells were infected at a multiplicity of 10 and harvested 48 h p.i. (A) Lane M, molecular weight markers; lane 1, VP1&2(cDNA)$_{AC}$; lane 2, VP2(cDNA)$_{AC}$; lane 3, VP1&2 (DNA)$_{AC}$; lane 4, wild-type AcNPV; lane 5, uninfected Sf9 cells. The polyhedrin gene product expressed exclusively by wild-type AcNPV is marked PH. (B) Immunoblot analysis with a pool of sera from infected mink as an antibody. Lane M, total lysates from ADV-G-infected CRFK cells; lane 1, VP1&2(cDNA)$_{AC}$; lane 2, VP2(cDNA)$_{AC}$; lane 3, VP1&2(DNA)$_{AC}$; lane 4, wild-type AcNPV; lane 5, uninfected Sf9 cells. The ADV structural proteins are marked VP-1 and VP-2, and the nonstructural protein observed in ADV-G-infected CRFK cells is marked NS-1.

context, mutation of a lysine to a serine in the nucleotide-binding pocket of the H-1 virus NS-1 protein has been shown to abolish the cytotoxicity mediated by the NS-1 protein (43). A similar mutation in the ADV NS-1 gene allowed high NS-1 expression and high recombinant AcNPV titer production (data not shown). The ADV gene product expressed from NS-2$_{AC}$ seems to be localized mainly in the cytoplasm of Sf9 cells, although the possibility that the staining is in the periphery of the nucleus, because of the sparse cytoplasm of the Sf9 cells, cannot be excluded. The gene product from NS-3$_{AC}$ had a more diffuse distribution throughout the cell. For both NS-2 and NS-3, the observed fluorescence when polyclonal antisera were used was very weak (Fig. 3D and E). NS-2 proteins expressed by other parvoviruses have been reported to be localized in both the cytoplasm and the nucleus (27).

In lysates from Sf9 cells infected with NS-2Long$_{AC}$, NS-2 and, surprisingly, VP1 and VP2 were produced (Fig. 7A and B, lanes 2). The NS-2Long gene contains the ORFs from both NS-2 and VP1&2, but the first ATG in this mRNA is in the start of the ORF for NS-2. Thus, expression of VP1-VP2 from this mRNA indicates either leaky scanning of the ribosome at the NS-2 ATG or internal initiation on the mRNA. The molar ratios of VP1 to VP2 are still reversed in comparison with ADV-G. The molar ratio of NS-2 to VP1-VP2 translated from NS-2Long is judged from SDS-PAGE and Western blot analysis (Fig. 7A and B, lanes 2) to be approximately 100:1, indicating that translation from the NS-2 ATG is carried out most efficiently.

DISCUSSION

The baculovirus expression system has several advantages. It is possible to easily express biological active proteins in large quantities. Furthermore, splicing of RNA and transcription from internal promoters in the inserted gene are very inefficient in baculovirus-infected cells, reducing the risk for expression of aberrant gene products (36, 42). In this report, we have described the expression of ADV proteins from gene constructs on the basis of our proposed model of ADV transcription and translation (5) by using a recombinant AcNPV vector in insect cells.

The ADV structural proteins VP1 and VP2 were expressed from three different gene constructs in recombinant AcNPVs. One of the recombinant AcNPVs [VP1&2 (cDNA)$_{AC}$] contained the ORFs and start ATGs for both VP1 and VP2, and we previously proposed (5) that both VP1 and VP2 could be expressed from this cDNA by a leaky scanning mechanism of the ribosome during translation (5). This is in contrast to other parvoviruses, in which VP1 and VP2 are translated from alternative spliced mRNAs (20, 37, 50). As predicted, both VP1 and VP2 were expressed from this gene, although the ratio of VP1 to VP2 was reversed from that seen during ADV-G infection in CRFK cells and CCL-64 cells (an embryonic mink lung cell line) (16). One explanation could be that the regulation of translation initiation was different in AcNPV-infected Sf9 insect cells and mammalian cells. However, this possibility seems unlikely because when the same gene construct is transiently expressed after transfection into COS-7 cells (data not shown) or expressed in CV-1 cells by a recombinant vaccinia virus (24), the reversed ratio of VP1 to VP2 is still observed. A more likely explanation could be, as suggested by Clemens et al. (24), that the native structure of the ADV R3 mRNA, which contains 380 residues 5′ to the VP1 initiation site and includes two minicistrons encoding 8 and 100 amino acids and containing five in-frame AUGs, somehow may regulate the initiation frequencies of VP1 and VP2 at the translational level. A similar mechanism has also been observed for VP1 in parvovirus B19 (49). Another explanation could be the existence of an alternative spliced ADV mRNA not identified during our cDNA cloning. However, polymerase chain reaction analysis of ADV cDNA around the mRNA splice junctions did not reveal any other splice pattern, making this possibility unlikely (22c). VP2 could be expressed from both VP2(cDNA)$_{AC}$ and VP1&2(DNA)$_{AC}$, confirming that VP2 is expressed from the predicted ORF. Furthermore, only VP2 was expressed from VP1&2(DNA)$_{AC}$, supporting the predicted ORF for VP1 and confirming that splicing in AcNPV-infected Sf9 cells is inefficient (36, 42).

Both VP1 and VP2 produced by recombinant AcNPVs were able to assemble into icosahedral 25-nm particles, and

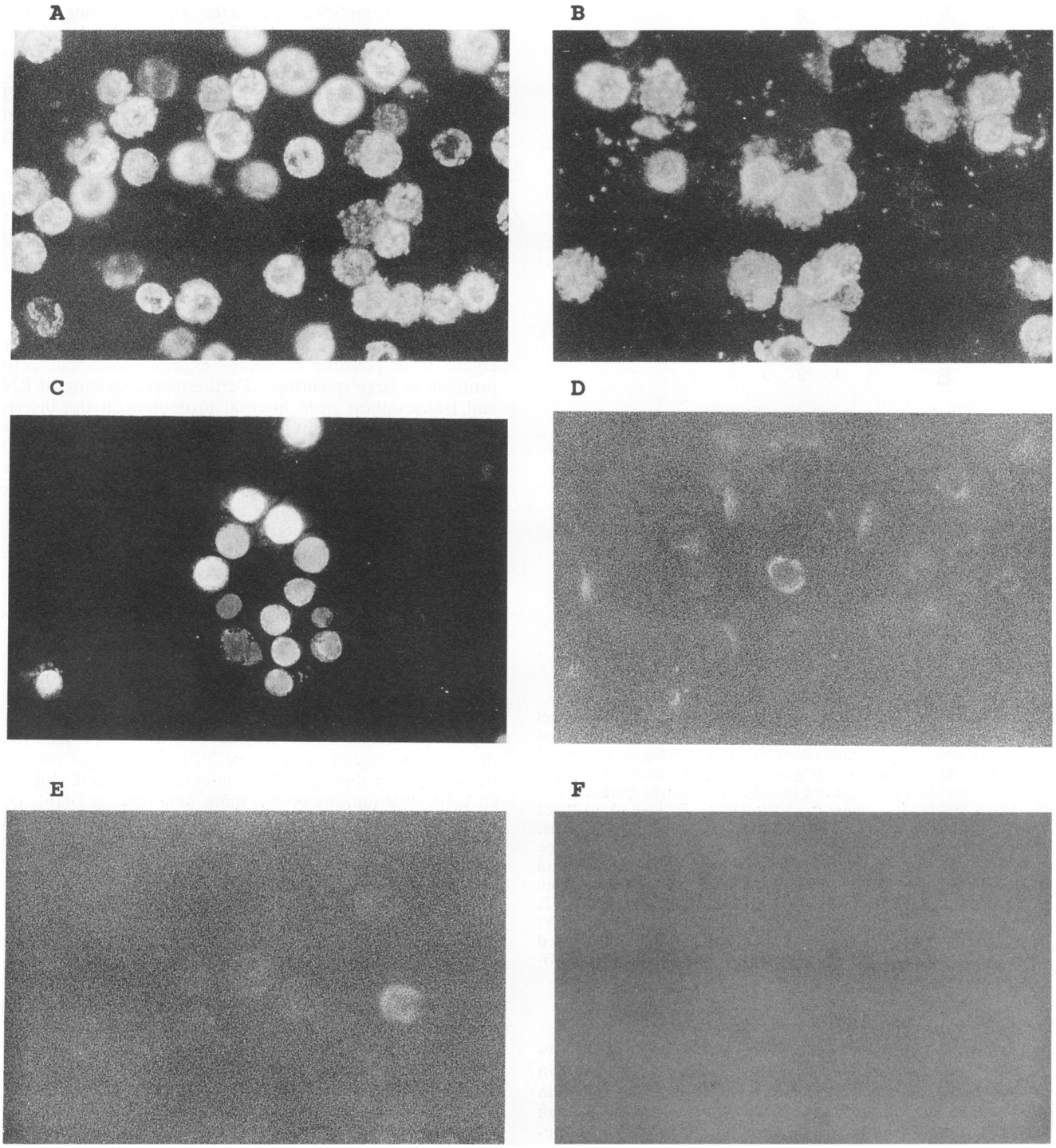

FIG. 3. Immunofluorescence analysis of Sf9 cells infected with recombinant AcNPVs 48 h p.i. (A) VP1&2(cDNA)$_{AC}$; (B) VP2(cDNA)$_{AC}$; (C) NS-1$_{AC}$; (D) NS-2$_{AC}$; (E) NS-3$_{AC}$; (F) control (Sf9 cells infected with recombinant polyhedrin-negative AcNPV expressing β-galactosidase).

the results suggested that particle formation is independent of any specific ratio of VP1 to VP2. The high VP1/VP2 ratio observed for ADV is in contrast to what is observed for parvovirus B19. In the B19 virus system, VP1 can be incorporated into the particles only together with a higher molar concentration of B19 virus VP2 (18, 38). Particle formation of VP2 alone has been shown for canine parvovirus, feline parvovirus, MVM (56, 63), and mink enteritis virus (22b), but data concerning VP1 in these cases are still lacking. The special feature of ADV, i.e., the ability to

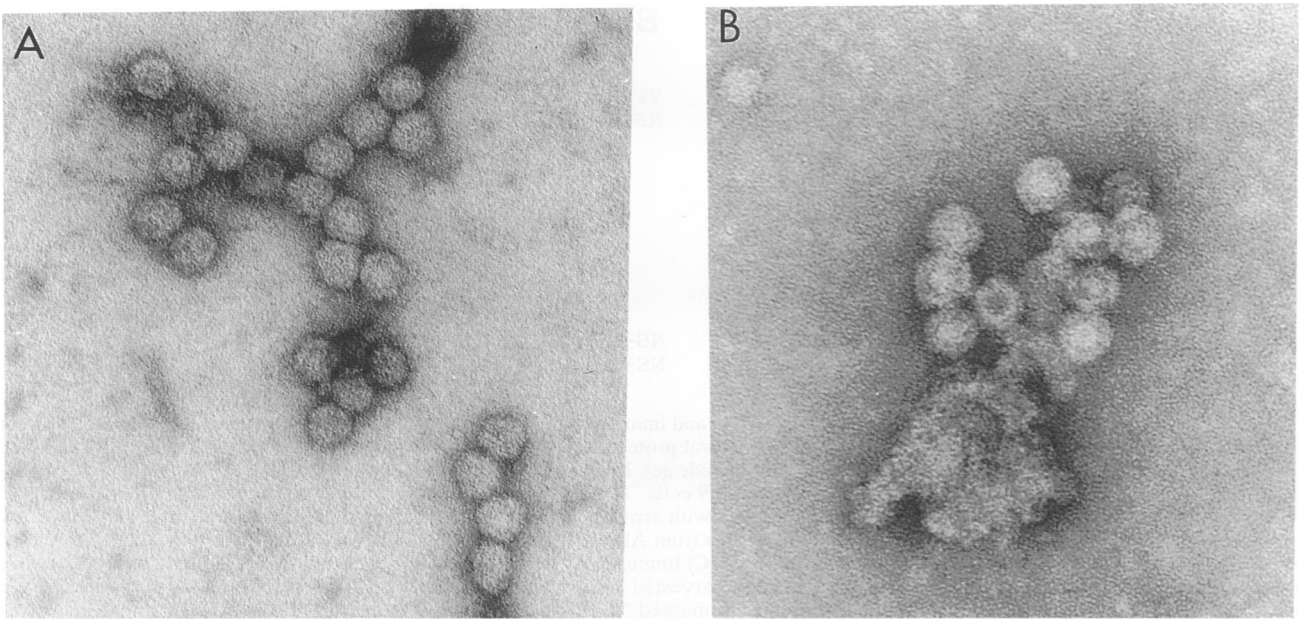

FIG. 4. Electron micrograph of negatively stained 25-nm ADV-like particles expressed by recombinant AcNPV. (A) VP1&2(cDNA)$_{AC}$; (B) VP2(cDNA)$_{AC}$. Magnification, ×260,000.

incorporate large amounts of VP1 into capsids, might increase the possibility of determining the three-dimensional organization of the VP1 molecule in the particle, provided that the amino terminus of the molecule has an ordered structure. Furthermore, this observation indicates that ADV might have a three-dimensional structure unique among the parvoviruses. Maybe the size of the VP2 molecule plays an important role, since ADV VP2 is approximately 80 amino acids larger than the VP2s of other parvoviruses. Also, the small difference in size between ADV VP1 and VP2 (only 44 amino acids compared with a difference of about 140 amino acids for the other parvoviruses) may be important. Interestingly, it has been shown that ADV particles found in vivo consist of structural proteins proteolytically processed to a size of 30 kDa, and moreover, such particles are still infectious in vivo (1a). This size range of the ADV structural proteins found in the particles might play a major role in the pathogenesis of ADV infection in mink. AcNPV-expressed VP1 and VP2 localized to the nuclei of Sf9 cells, suggesting that VP1 and VP2 can be transported independently into Sf9 nuclei, probably by using a common nuclear localization signal located in their overlapping amino acid sequence.

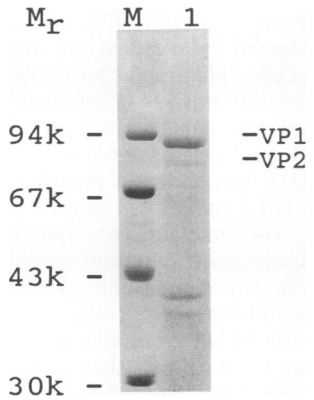

FIG. 5. Purified recombinant AcNPV-expressed ADV-like particles analyzed by SDS-PAGE. ADV-like particles were purified from recombinant AcNPV-infected Sf9 cells. The cells were sonicated, and cell debris was removed by low-speed centrifugation followed by ultracentrifugation of the supernatant through a 40% sucrose cushion. Lane M, molecular weight markers; lane 1, particles expressed by VP1&2(cDNA)$_{AC}$. The ADV structural proteins are marked VP1 and VP2.

The antigenicity and immunogenicity of the ADV-like particles generated from recombinant AcNPV seem to be promising, and these particles might be an important diagnostic reagent for eradication of ADV infection in mink. Studies of this possibility are in progress.

The predicted ADV NS-1 ORF was constructed by cloning a combination of cDNA and genomic ADV DNA into AcNPV. Analysis of the gene product showed several features expected for the ADV NS-1 protein. It had a molecular weight similar to that of NS-1 expressed in cells infected with the ADV-G isolate. A nuclear localization in Sf9 cells and in transiently transfected cell lines was also observed. In this regard, the nuclear localization signal of ADV NS-1 is apparently different from the one encoded by MVM, since MVM NS-1 is not localized in the nucleus when expressed by a recombinant baculovirus in Sf9 cells (65).

The constructed ADV NS-1 gene is able to transactivate parvovirus promoters and down regulate heterologous promoters when tested by transient transfection in different cell lines (22a), consistent with observations for other parvoviruses (29, 30, 54, 55). The cytotoxic or cytostatic properties of the NS-1 gene product observed here are also in agreement with several other reports (19, 25, 33, 43, 48). Although the mechanism of the cytotoxicity mediated by NS-1 is still not fully resolved, this effect on the host cell could at least in part be explained by the observations of Ho et al. (34). They observed that during MVM infection in cell culture, the primase normally associated with DNA polymerase α is

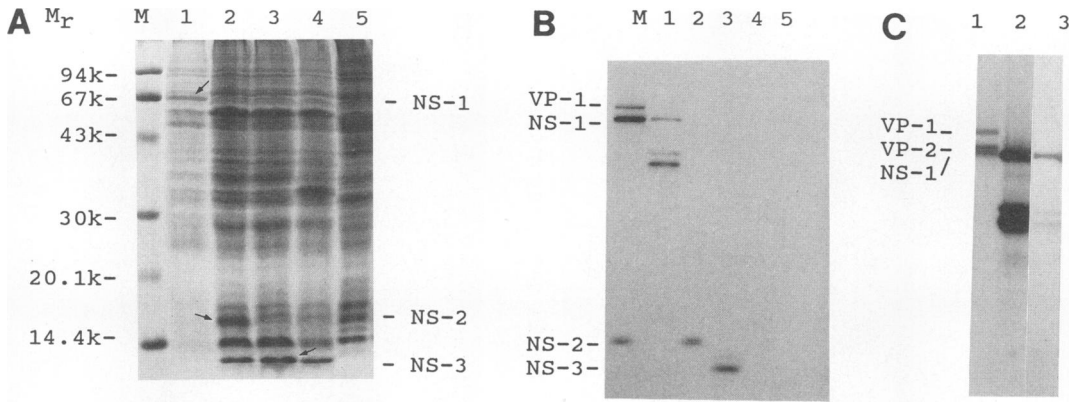

FIG. 6. Coomassie blue-stained SDS-polyacrylamide gel (A) and immunoblot (B and C) analysis of total lysates of Sf9 cells infected with three different recombinant AcNPVs expressing the nonstructural proteins of ADV. The Sf9 cells were infected at a multiplicity of 10 and harvested 48 h p.i. (A) Coomassie blue-stained 15% polyacrylamide gel. Lane M, molecular weight markers; lane 1, NS-1_{AC}; lane 2, NS-2_{AC}; lane 3, NS-3_{AC}; lane 4, wild-type AcNPV; lane 5, uninfected Sf9 cells. The NS-1, NS-2, and NS-3 bands comigrate with other bands in the Sf9 lysates; the positions of NS-1, NS-2, and NS-3 are marked with arrows. (B) Immunoblot analysis (15% polyacrylamide gel) with a pool of sera from infected mink as an antibody. Lane M, total lysates from ADV-G-infected CRFK cells; lane 1, NS-1_{AC}; lane 2, NS-2_{AC}; lane 3, NS-3_{AC}; lane 4, wild-type AcNPV; lane 5, uninfected Sf9 cells. (C) Immunoblot analysis (10% polyacrylamide gel). Lane 1, total lysates from ADV-G-infected CRFK cells; lane 2, NS-1_{AC}; lane 3, NS-1_{AC} harvested in the presence of protease inhibitors. The ADV structural proteins are marked VP-1 and VP-2, and the nonstructural proteins are marked NS-1, NS-2, and NS-3.

absent in the host cell complex of DNA replication proteins. This situation is quite favorable for parvovirus DNA replication because it would avoid lagging strand synthesis during progeny single-stranded DNA production from double-stranded replicative-form DNA. At the same time, the absence of lagging strand synthesis wreaks havoc on the host cell genome. Ho et al. (34) have suggested that NS-1 may be noncovalently associated with DNA polymerase α. The interaction of NS-1 and DNA polymerase α would be very similar to the noncovalent association of simian virus 40 large T antigen and DNA polymerase α (31). The consequence of such a mechanism for the host cell would be the continuous presence of partly replicated chromosomal DNA, which most likely would disrupt chromatin structure and inhibit exit from the S phase and entry into mitosis. This is in agreement with the observed induction of cell cycle arrest in the late S/G_2 phase of the cell cycle (9, 10) and the destruction of chromatin structure caused by parvovirus infection (57, 58). Furthermore, this situation would favor prolonged replication of the parvovirus DNA, because the presence of single-stranded regions in the partially replicated chromosomal DNA would prevent host shutoff of DNA replication (59). Parvoviruses are dependent on S-phase factors in dividing cells and cannot stimulate the cells to enter the S phase (26).

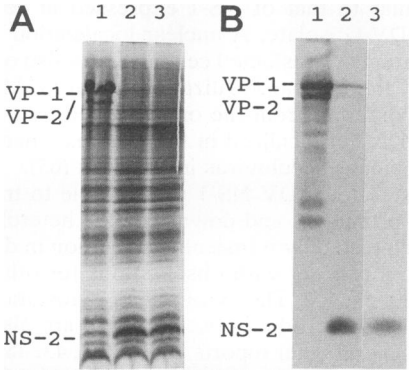

FIG. 7. Analysis of the expression pattern of the NS-2Long gene by SDS-PAGE (A) and immunoblotting (B) (15% polyacrylamide gels). Total lysates of Sf9 cells infected with recombinant AcNPV were analyzed 48 h p.i. Lanes 1, VP1&2(cDNA)$_{AC}$; lanes 2, NS-2Long$_{AC}$; lanes 3, NS-2_{AC}.

Expression of the NS-2 gene by recombinant AcNPV allowed alignment of this gene product with a similar product seen in ADV-G-infected cell cultures. The mRNA coding for NS-2 has also been demonstrated to be present in vivo (59a) and is the most abundant mRNA in cell culture (5). The NS-2 and NS-3 proteins are recognized by sera from infected mink. However, whether these antibodies specifically are directed against NS-2 and NS-3 or cross-react with the common amino-terminal amino acid sequence also present in NS-1 remains to be answered. The NS-3 protein could be expressed by recombinant AcNPV, but no protein of that size could be detected by Western blot analysis of ADV-G-infected CRFK cells. This might be explained by the very low abundance of this mRNA in infected cell cultures (5). The functions of the smaller NS proteins during ADV infection have not yet been resolved, but analysis of different field types of ADV has shown a preservation of the NS-2 gene, while the NS-3 product in one of the ADV types is truncated (32a). This may indicate that the NS-2 protein probably has an important function, while NS-3, at least in some situations, may be dispensable. ADV NS-2 seems to be located in the cytoplasm of Sf9 cells, in contrast to what is reported for the MVM NS-2 proteins in infected mammalian cells (27). It has been shown that in the MVM system, phosphorylated NS-2 is confined to the cytoplasm, while unphosphorylated NS-2 is located in the nucleus and cytoplasm. In fact, ADV NS-2 migrates with a larger size than expected, perhaps because of posttranslational modifications such as phosphorylation (25, 27); this could explain the cytoplasmic localization. Two different mRNAs transcribed from the P3 promoter during ADV-G infection in cell culture can code for NS-2 (5). The most abundant form is a short

version polyadenylated in the middle of the genome. A less abundant, and longer, form designated NS-2Long uses a polyadenylation signal near the right end of the genome and contains the coding capacity for VP1 and VP2 in addition to that for NS-2. Because B19 virus transcribes all genes including VP1 and VP2 from the left promoter (50), it was of interest to investigate the expressed proteins from the NS-2Long gene. Interestingly, NS-2 and also, albeit at low levels, VP1 and VP2 were expressed. The ratio of VP1 to VP2 is still reversed from that of ADV-G infection in cell culture. It has been reported for MVM that the 5′ end of RNA transcribed from the left promoter can form stem-loop conformations (attenuator conformation or readthrough conformation) involving the AUG for the nonstructural proteins and thus could influence translation (39). In the attenuator conformation, the AUG used by NS-2 and NS-1 is available for translation initiation, while in the readthrough conformation it is potentially sequestered in the stem. However, whether the expression pattern from this NS-2Long mRNA has any biological significance for ADV infection or is a feature of the AcNPV expression system remains to be resolved.

In conclusion, our results supported the proposed transcriptional and translational program of ADV and allowed a preliminary characterization of the viral proteins expressed during ADV infection. The ADV-encoded proteins can now be produced in milligram quantities, allowing a detailed further biochemical analysis of their biological functions. Such a study is in progress.

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