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Purification and Characterization of the Major Nonstructural Protein (NS-1) of Aleutian Mink Disease Parvovirus

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We have previously described the expression of the major nonstructural protein (NS-1) of Aleutian mink disease parvovirus (ADV) in insect cells by using a baculovirus vector (J. Christensen, T. Storgaard, B. Bloch, S. Alexandersen, and B. Aasted, J. Virol. 67:229–238, 1993). To study its biochemical properties, ADV NS-1 was expressed in Sf9 insect cells and purified to apparent homogeneity with a combination of nuclear extraction, Zn^{2+} ion chromatography, and immunoaffinity chromatography on monoclonal antibodies. The purified protein showed ATP binding and ATPase- and ATP- or dATP-dependent helicase activity requiring either Mg^{2+} or Mn^{2+} as a cofactor. The ATPase activity of NS-1 was efficiently stimulated by single-stranded DNA and, to a lesser extent, double-stranded DNA. We also describe the expression, purification, and characterization of a mutant NS-1 protein, in which a lysine in the putative nucleotide binding consensus sequence of the molecule was replaced with serine. The mutated NS-1 was expressed at 10-fold higher levels than wild-type NS-1, but it exhibited no ATP binding, ATPase, or helicase activity. The availability of large amounts of purified functional NS-1 protein will facilitate studies of the biochemistry of ADV replication and gene regulation leading to disease in mink.

Aleutian mink disease parvovirus (ADV) is an autonomous parvovirus and is an important cause of disease in mink (44). No effective vaccines have been developed. The disease pattern ranges from acute pneumonia in mink kits not protected by maternal antibodies to persistent infection of adult mink resulting in chronic immune complex disease, often with a fatal outcome (2, 3, 5, 24, 43, 45).

Parvoviruses are small single-stranded DNA viruses with genome sizes in the range of 5 kb. The vertebrate parvoviruses are characterized by a similar genomic organization, and the coding regions are confined to the plus strand (7, 18). Because of the small size of the genome, parvoviruses use an extensive repertoire of genetic strategies to enhance their coding potential. These include the use of multiple promoters and overlapping reading frames, as well as alternative mRNA splicing, alternate polyadenylation signals, posttranslational modification, and multifunctional proteins (7, 18, 32, 42). The genome can basically be divided into two parts, with the left-hand region encoding two to four nonstructural proteins and the right-hand side coding for the virus structural proteins (4, 7, 13, 17, 32).

Parvovirus DNA replication takes place in the cell nucleus and is strictly dependent on the biochemical activities of the major virus nonstructural protein(s), designated NS-1 in the autonomous parvoviruses and Rep in the adeno-associated viruses (AAVs) and on factors expressed in the S phase of dividing cells (8, 18). The NS-1 protein is a multifunctional nuclear phosphoprotein which is absolutely required for parvovirus replication both in vivo and in vitro (13, 15, 16, 20, 21, 40). Functional analyses have shown that NS-1 and Rep are sequence-specific DNA binding proteins which have intrinsic ATPase, helicase, and site-specific endonuclease activities (10, 14, 29, 36, 37, 53). These activities are involved in the catalysis of nicking, covalent attachment of NS-1 to the 5′ end of the viral genome, resolution of viral telomers and concatemeric intermediates, and generation of single-stranded progeny DNA during virus replication (15, 16, 19, 29, 40, 47). Furthermore, NS-1 is a transactivator of parvovirus transcription. In contrast, NS-1 downregulates a variety of other viral and cellular promoters (12, 22, 23, 46). NS-1 and simian virus 40 (SV40) large T antigen (TAg) show sequence homology in certain regions of the protein (6). Both proteins have an A-type purine nucleotide binding consensus sequence [(G/A)X_4GK(S/T)X_{5-6}I/L/V] and share a number of biological activities involved in virus replication and transcription. However, in contrast to TAg, NS-1 exerts a cytotoxic/cytostatic effect on the host cell (9, 46) and is unable to induce proliferation of quiescent cells.

In this study, we describe the production of murine monoclonal antibodies (MAbs) to NS-1 and the development of an immunoaffinity purification procedure for NS-1 expressed in insect cells and characterize the intrinsic enzymatic activities of this purified NS-1. This will allow future functional studies of NS-1 in vitro. Furthermore, we have purified and characterized an NS-1 mutant in which a lysine in the putative A-type purine nucleotide sequence has been mutated into a serine. This substitution has been shown to abolish most of the biological functions of NS-1 (34). Here, we demonstrate that this mutation severely affects a number of the protein's intrinsic enzymatic activities.

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MATERIALS AND METHODS

Chemicals and reagents. Nucleotides and ribonucleotides were obtained from Pharmacia (Uppsala, Sweden) or Promega Corp. (Madison, Wis.). ^{32}P-labeled ATP or dATP and ^{125}I-protein A were obtained from Amersham. Protein G-

Sepharose was purchased from Pierce (Rockford, Ill.). Protamine sulfate (P-4020) was obtained from Sigma (St. Louis, Mo.).

Cells and viruses. *Spodoptera frugiperda* (Sf9) insect cells and the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) were obtained from In Vitrogen (San Diego, Calif.). Cells were cultured in Grace medium supplemented with 10% fetal calf serum, yeastolate, 0.15% pluronic F-68 (Sigma), and 50 μg of gentamicin in spinner bottles at 28°C. For expression of recombinant protein, 7.5×10^7 Sf9 cells were plated in tissue culture dishes (245 by 245 mm) in medium without pluronic F-68 and infected at a multiplicity of infection (MOI) of between 1 and 10.

Antisera. The antiserum reactive to ADV structural and nonstructural proteins was a pool of sera from ADV-infected mink.

SDS-PAGE and immunoblots. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblots were performed as previously described (11).

Construction and purification of rAcNPVs. Isolation of a recombinant baculovirus (rAcNPV) harboring ADV nucleotides 196 to 1961, 2042 to 2213, and 2287 to 2353 expressing ADV NS-1 has been described previously (11). Mutation of a lysine to a serine in the putative purine binding pocket of ADV NS-1 at amino acid 438 (designated NS-1K438S) by site-directed mutagenesis of the ADV NS-1 gene and cloning of the mutated gene into the pSVL vector (pSVL NS-1K438S) has also been described previously (12). To generate recombinant baculoviruses expressing the NS-1K438S gene, the mutated NS-1 gene was cloned as an *Xba*I fragment derived from the pSVL NS-1K438S plasmid into the *Nhe*I cloning site of the pBlueBac βII (In Vitrogen) baculovirus transfer vector. Recombinant baculoviruses were isolated from supernatants of Sf9 cells cotransfected with the baculovirus transfer vector containing the NS-1K438S gene and wild-type baculovirus DNA as previously described (11).

Isolation of hybridomas. Hybridomas were generated by a standard protocol. NS-1K438S was expressed in Sf9 insect cells and purified by salt nuclear extraction and Zn^{2+} ion-affinity chromatography. For immunization, two female BALB/c mice were immunized intraperitoneally with 50 μg of purified NS-1K438S in Freund's incomplete adjuvant. After 3 weeks, the mice were given intraperitoneal booster injections of 50 μg of NS-1K438S in Freund's incomplete adjuvant, and a week later the mice were tested for antibody to NS-1K438S with an enzyme-linked immunosorbent assay (ELISA). After a second boost with 50 μg of NS-1K438S in phosphate-buffered saline adjusted to 300 mM NaCl, splenocytes from one mouse were fused to X63-Ag8.653 cells (33) and hybridomas were selected with hypoxanthine-aminopterin-thymidine medium. The hybridomas were cultured in RPMI 1640 medium with Glutamax-1 (Life Technologies, Roskilde, Denmark), supplemented with 15% fetal calf serum and penicillin and streptomycin as antibiotics, at 37°C in a 5% CO_2 humidified atmosphere. Four hybridomas expressing antibodies which reacted with ADV NS-1 in the ELISA were obtained (MAb505, MAb507, MAb511, and MAb415), subcloned three times by limiting dilution, and stored under liquid nitrogen. The MAbs were subtyped with a Mono Ab-ID enzyme immunoassay mouse kit (Zymed, San Francisco, Calif.). The four MAbs were all of the mouse immunoglobulin G1 (IgG1) subtype.

Purification of MAbs and preparation of immunoaffinity columns. The hybridoma supernatants were slowly adjusted to approximately 50% saturation with ammonium sulfate by addition of 300 mg of solid $(NH_4)_2SO_4$ per ml of supernatant with constant stirring at 4°C. The pH was maintained at 7.4 by addition of NaOH. After 30 min, the precipitated proteins were collected by centrifugation at 20,000 × *g* for 45 min and dissolved in a minimal volume of 20 mM Tris-HCl (pH 8.0)–1 mM EDTA. The remaining ammonium sulfate was removed by extensive dialysis against 0.1 M sodium acetate buffer (pH 5.2). The IgGs were loaded on a column of protein G-Sepharose that was equilibrated with the dialysis buffer. The column was extensively washed, and the IgGs were eluted with a 0.1 M glycine-HCl buffer (pH 2.7). The eluate was immediately neutralized with 1 M Tris base. The purified MAbs were tested for purity by SDS-PAGE. The purified MAbs were then immobilized on cyanogen bromide-activated Sepharose 4b (Pharmacia) at a concentration of 5 mg of IgG per ml of gel according to the manufacturer's description. Typically, more than 95% of the antibodies bound to the column material.

Purification of recombinant NS-1 and the NS-1K438S mutant. For expression of recombinant protein, 7.5×10^7 Sf9 cells were plated in tissue culture dishes (245 by 245 mm) in medium without pluronic F-68. For NS-1 expression, Sf9 cells were infected at a MOI of 1 to 2. The low MOI used is due to the difficulty in obtaining high-titer stocks of baculovirus expressing NS-1, probably because the protein is cytotoxic. Thus, titers of wild-type NS-1-expressing virus were often less than 10^7 infectious doses (IDs) per ml. In contrast, it was relatively easy to obtain high-titer stocks of a baculovirus expressing the NS-1K438S mutant gene (higher than 10^8 IDs/ml), and for this mutant, the Sf9 cells were infected at a MOI of 5 to 10. Cells were harvested 40 to 48 h postinfection by being scraped into the medium, followed by centrifugation (500 × *g* for 5 min at 4°C). The purification scheme consisted essentially of three steps: a nuclear salt extraction procedure, Zn^{2+} ion-affinity chromatography, and immunoaffinity chromatography. All procedures were carried out on ice or at 4°C. For nuclear extraction, 3×10^8 Sf9 cells were washed three times in ice-cold TNE (40 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA). To isolate Sf9 nuclei, the washed cells were resuspended in 15 ml of lysis buffer (TNE, 0.3% Nonidet P-40, 1 mM dithiothreitol [DTT], 8 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride) and incubated for 10 min. The nuclei were pelleted by centrifugation at 800 × *g* for 10 min and washed twice in 15 ml of lysis buffer without Nonidet P-40. The isolated nuclei were resuspended in 7 ml of lysis buffer without Nonidet P-40 and adjusted to 350 mM NaCl. After incubation for 30 min to extract the NS-1 protein, the nuclei were removed by centrifugation at 10,000 × *g* for 10 min. To remove baculovirus DNA coextracted during the nucleus extraction procedure, the supernatant was adjusted to 2 mg of protamine sulfate (with a 20-mg/ml stock solution of protamine sulfate) and centrifuged for 30 min at 20,000 × *g*. For Zn^{2+} ion-affinity chromatography, 2 ml of chelating Sepharose Fast Flow (Pharmacia) was loaded with Zn^{2+}, poured into a small column, and equilibrated with buffer A (25 mM Tris-HCl) [pH 7.5], 300 mM NaCl, 0.1 mM DTT, 4 μg of leupeptin per ml). The nuclear extract was applied to the column through a precolumn of 1 ml of chelating Sepharose without immobilized Zn^{2+}. After a washing with 15 ml of buffer A, the column was washed with 15 ml of buffer A adjusted to 800 mM NaCl and then rewashed with 15 ml of buffer A. The column was eluted with 10 ml of buffer A adjusted to 20 mM EDTA. For immunoaffinity chromatography, the eluate from the Zn^{2+} ion-affinity chromatography column was adjusted to 0.15% Nonidet P-40 and 10% glycerol and absorbed in batch suspension to 2 ml of MAb415-coupled Sepharose equilibrated in buffer B (buffer A adjusted to 0.15% Nonidet P-40 and 10% glycerol). The MAb415 matrix was pelleted by centrifugation and resuspended in a small volume of buffer B and poured into a small column. The column was washed with 10 volumes of buffer B adjusted to 600 mM NaCl, followed by 5 volumes of buffer C (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 9.0], 300 mM NaCl, 0.1 mM DTT, 4 μg of leupeptin per ml, 10% glycerol). The column was eluted with 20 mM triethylamine (pH 11.3)–0.3 M NaCl–4 μg of leupeptin per ml–10% glycerol and immediately neutralized with 1/10th volume of 1 M HEPES-KOH (pH 7.0)–300 mM NaCl–10% glycerol. The fractions were dialyzed overnight against 25 mM Tris-HCl (pH 7.5)–300 mM NaCl–0.1 mM DTT–2 μg of leupeptin per ml–20% glycerol, and purified preparations were stored at −80°C.

Purification of SV40 large TAg. SV40 TAg was expressed in Sf9 insect cells and immunoaffinity purified with the MAb Pab101 coupled to cyanogen bromide-activated Sepharose 4b as described previously (27).

ATPase assay. The ATPase assay was performed essentially as described by Wilson et al. (53). Briefly, different concentrations of the purified protein preparations were incubated at 37°C for 15 min in 25 mM Tris-HCl (pH 7.5)–100 mM NaCl–5 mM $MgCl_2$–5 mM DTT–0.01% Nonidet P-40–30 μM unlabeled ATP–0.5 μCi of [^{32}P]ATP (3,000 Ci/mmol). The reaction was terminated by addition of 100 μl of 7.5% (wt/vol) acid-washed charcoal in 5 mM H_3PO_4. After being pelleted by centrifugation, 80 μl of the supernatant was analyzed for $^{32}P_i$ by Cerenkov counting.

ATP binding assay. The ATP binding assay was performed with a spin column assay as described for the yeast (*Saccharomyces cerevisiae*) RAD3 protein (51). Four micrograms of NS-1 or NS-1K438S was incubated in 20 mM Tris-HCl (pH 7.5)–300 mM NaCl–0.01% Nonidet P-40, 120 μg of bovine serum albumin (BSA) per ml–7 mM $MgCl_2$–0.1 μCi of [^{32}P]ATP (3,000 Ci/mmol)–0.0712 pM ATP in a total volume of 80 μl for 30 min on ice. The ATP associated with NS-1 was separated from unbound nucleotide with a 1-ml Sephadex G-50 spin column equilibrated with the buffer described above without ATP at 4°C. For evaluation of the specificity of the assay, the incubations were performed in the presence of either 0.125 mM ATP or 0.125 mM CTP.

Helicase assay. The helicase assay was performed essentially as described in reference 53. Briefly, the NS-1 preparations were incubated for 1 h at 37°C in a buffer containing 20 mM Tris-HCl (pH 7.5)–5 mM $MgCl_2$–5 mM DTT–4 mM ATP–0.1 mg of BSA per ml–(2 to 4) × 10^4 cpm of a 26-nucleotide ^{32}P-labeled oligomer annealed to M13mp18 single-stranded (ss) DNA (produced from components of the Sequenase version 2.0 DNA sequencing kit; U. S. Biochemical Corp., Cleveland, Ohio). After incubation, the reaction mixtures were analyzed by electrophoresis on a nondenaturing 6% polyacrylamide gel. The gels were dried and exposed for autoradiography.

RESULTS

Expression of NS-1 and NS-1K438S. We have shown previously when the ADV NS-1 protein is expressed in insect cells from a baculovirus vector (designated $NS\text{-}1_{AC}$) that expression levels are low compared with those achieved for other ADV-encoded proteins, suggesting that the NS-1 molecule is somewhat cytotoxic in these cells. Furthermore, it was difficult to obtain high titer stocks of rAcNPV expressing ADV NS-1 (titers were less than 10^7 IDs/ml) (11). It has been reported that mutations in the putative purine binding consensus sequence of NS-1 can reduce the cytotoxic action of the protein to the host cell (34). Therefore, to achieve a high level of expression in the insect cell system, we mutated a lysine at amino acid 438 in this consensus sequence of the ADV NS-1 gene to a serine (designated NS-1K438S). To examine the

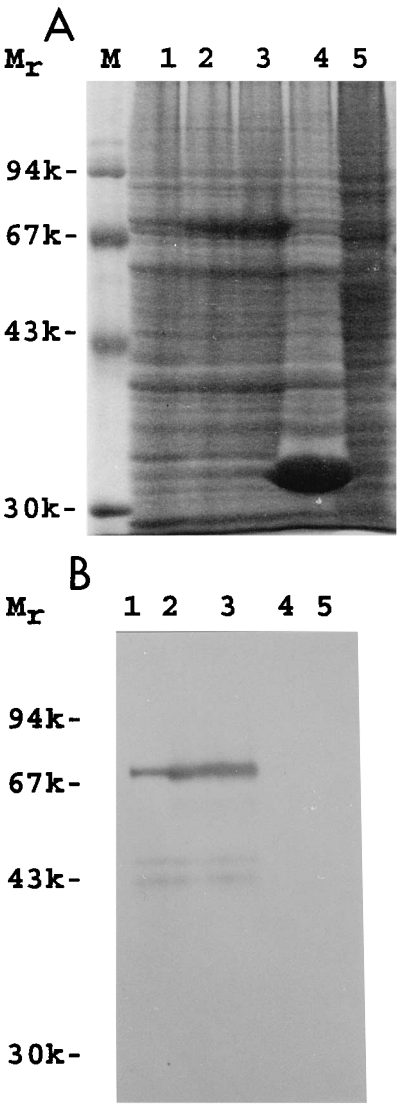

FIG. 1. SDS-PAGE and immunoblot analysis of total lysates of Sf9 cells infected with recombinant baculoviruses expressing NS-1 and NS-1K438S. (A) Lanes: M, molecular weight markers; 1, NS-1_{AC}; 2 and 3, NS-1K438S_{AC} (two different isolates); 4, wild-type AcNPV; 5, uninfected Sf9 cells (the gel was stained with Coomassie brilliant blue dye). (B) Immunoblot analysis with a pool of sera from an ADV-infected mink as antibody. Lanes: 1, NS-1_{AC}; 2 and 3, NS-1K438S_{AC} (two different isolates); 4, wild-type AcNPV; 5; uninfected Sf9 cells.

expression of ADV NS-1K438S in insect cells, several rAcNPVs were purified and amplified. In contrast to the results obtained for expression of ADV NS-1, high-titer stocks of rAcNPV expressing ADV NS-1K438S (designated NS-1K438S_{AC}) were obtained easily and routinely exceeded 10^8 IDs/ml. SDS-PAGE and Western blot (immunoblot) analysis of total lysates of Sf9 insect cell monolayers infected at high MOIs (~10) and harvested 44 h postinfection showed high levels of NS-1K438S expression compared with the expression of ADV NS-1 (Fig. 1A and B, lanes 1, 2, and 3). The expression levels of NS-1K438S observed over multiple experiments were in the range of 5- to 20-fold higher than those of wild-type NS-1. Analysis of the Coomassie blue-stained gel by laser densitometry suggested that between 5 and 10% of the total protein in the NS-1K438S lysates was recombinant protein, corresponding to 5 to 10 mg/10^9 Sf9 cells. The minor bands observed for NS-1_{AC} and NS-1K438S_{AC} observed in the Western blot analysis are proteolytic degradation products of NS-1, because, as described earlier (11), the intensities of these bands are variable, depending on whether or not protease inhibitors are used and how the preparations are handled. Immunofluorescence analysis of Sf9 cells infected with NS-1K438S_{AC} showed that this mutant protein localized to the nuclei of Sf9 cells (data not shown), as observed earlier for the expression of NS-1_{AC} (11). The cytopathic effect of NS-1K438S_{AC} on Sf9 cells differed from that of NS-1_{AC}. While NS-1_{AC} infection slowly results in a decrease in the size of the nucleus in infected cells, NS-1K438S_{AC} infection of Sf9 cells results in the common increase of the nuclei as observed for wild-type baculovirus.

Purification of NS-1 and NS-1K438S and production of anti-NS-1 MAbs. In order to purify NS-1 and NS-1K438S, a three-step purification scheme was developed. The purification procedure consisted of a nucleus salt extraction procedure, Zn^{2+} ion-affinity chromatography, and, finally, immunoaffinity chromatography. Because it was known from the immunofluorescence analysis of NS-1_{AC}- and NS-1K438S_{AC}-infected cells that NS-1 and NS-1K438S localized to the nuclei of infected Sf9 cells, NS-1 and NS-1K438S were purified by extraction of isolated nuclei of NS-1_{AC}- and NS-1K438S_{AC}-infected Sf9 cells with NaCl (Fig. 2). Contaminating baculovirus DNA coeluting from the salt-extracted nuclei was removed with protamine sulfate. To further purify NS-1 and NS-1K438S, the nuclear extract was fractionated by Zn^{2+} ion-affinity chromatography. After extensive washing of the Zn^{2+} column, NS-1 and NS-1K438S were eluted with 20 mM EDTA (Fig. 2A and B, lane 5). At this purification step, NS-1 and NS-1K438S already had a high level of purity as judged by SDS-PAGE. (The two minor bands are degradation products of NS-1, as observed by Western blotting analysis.) However, contaminating bands of lower molecular weight were occasionally observed. To overcome this, we produced murine MAbs against purified NS-1K438S and immunoaffinity purified the NS-1 and NS-1K438S preparations. Four hybridomas (MAb505, MAb507, MAb511, and MAb415) expressing antibodies directed against NS-1K438S were identified by screening the hybridoma supernatants with an ELISA. All hybridomas secreted antibodies of the IgG1 subtype which gave nuclear staining in immunofluorescence analysis of ADV-G-infected CRFK cells or lung cryo-sections of ADV-Utah-infected mink kits (data not shown). ELISA analysis of the four MAbs indicated that MAb505, MAb507, and MAb511 reacted either with the same epitope or with overlapping epitopes, while MAb415 was directed against another site. In contrast to the other MAbs, MAb415 was active in NS-1 immunoprecipitation assays (data not shown). MAb415 was purified by protein G affinity chromatography from hybridoma culture supernatants and immobilized on cyanogen bromide-activated Sepharose. Eluates from Zn^{2+} ion-affinity columns were batch absorbed onto the immunoaffinity matrix, which was then poured into a small column and washed extensively. Finally, the immunoaffinity column was eluted and the fractions were immediately neutralized and dialyzed overnight against storage buffer (Fig. 2A and B, lane 6). The dialyzed fractions were stored at −80°C.

The yields of purified NS-1K438S and NS-1 in the eluate of the Zn^{2+} ion-affinity column were routinely 5 to 10 and 0.5 to 1 mg, respectively. Losses of NS-1K438S and NS-1 are estimated to be less than 10% during the nucleus extraction step and are of the same order of magnitude for the Zn^{2+} ion-affinity step. The yields after immunopurification depend on the binding capacity on the antibody matrix. Approximately 1 mg of NS-1 or NS-1K438S could be eluted from 1 ml of matrix

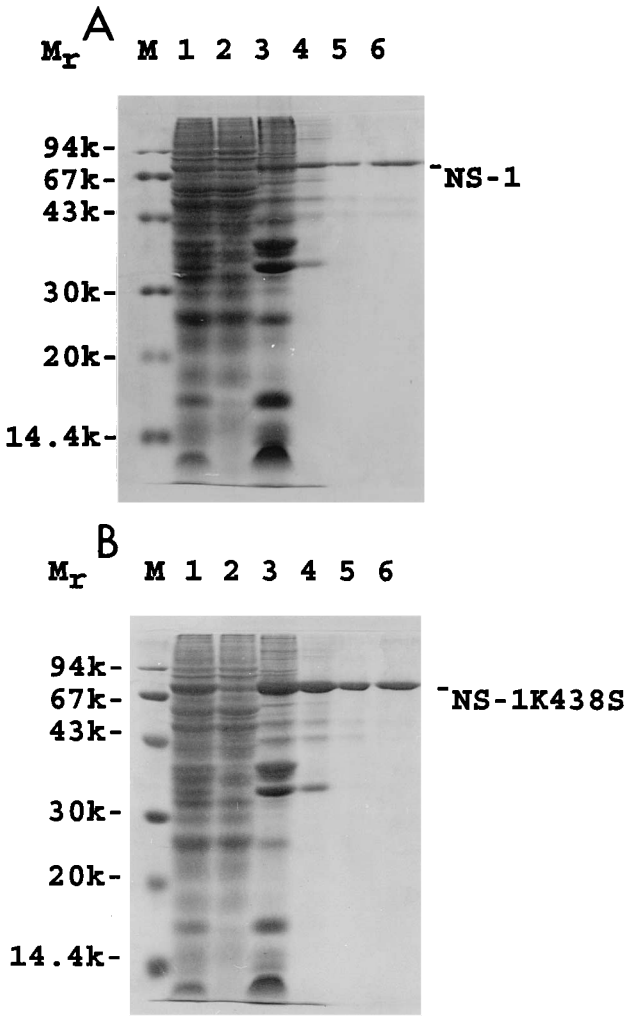

FIG. 2. SDS-PAGE analysis of purified fractions of NS-1 and NS-1K438S. (A) NS-1 fractions. Lanes: M, molecular weight marker; 1, total lysates of Sf9 cells infected with $NS\text{-}1_{AC}$; 2, cytoplasmic fraction; 3, nuclear fraction; 4, nuclear extract; 5, eluate from Zn^{2+} ion-affinity column; 6, 4 μg of the eluate from the immunoaffinity column. (B) NS-1K438S fractions. Lanes: M, molecular weight marker; 1, total lysates of Sf9 cells infected with $NS\text{-}1K438S_{AC}$; 2, cytoplasmic fraction; 3, nuclear fraction; 4, nuclear extract; 5, eluate from Zn^{2+} ion-affinity column; 6, 4 μg of the eluate from the immunoaffinity column. Protein concentrations of NS-1 were calculated from the optical densities at 280 nm, assuming that an optical density of 1 corresponds to an NS-1 concentration of 1 mg/ml. The gel was stained with Coomassie brilliant blue dye.

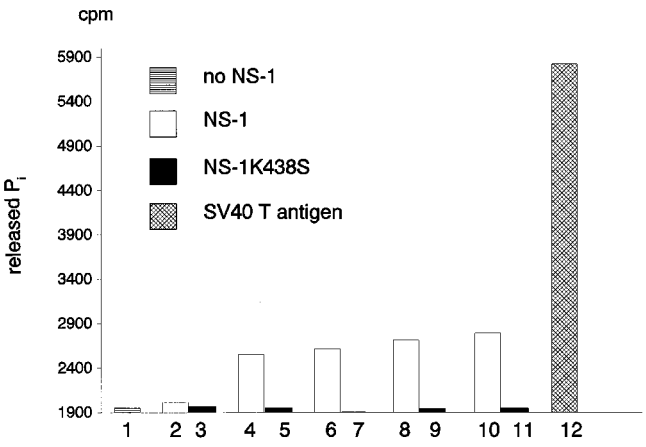

FIG. 3. Comparison of ATPase activity of immunopurified NS-1 and NS-1K438S. Bars: 1, no NS-1; 2, 4, 6, 8, and 10, 25, 50, 100, 150, and 200 ng of NS-1, respectively; 3, 5, 7, 9, and 11, 25, 50, 100, 150, and 200 ng of NS-1K438S, respectively; 12, 200 ng of SV40 TAg. The specific activity of the ATP was 0.67 mCi/mmol. The values are calculated as the means of duplicate determinations that generally differed less than 10%.

containing 5 mg of antibody. Dialysis of the eluted material after elution from the immunoaffinity column resulted in losses on the order of 30%. Comparison of the helicase activity of NS-1 eluted from the Zn^{2+} ion column with that of NS-1 eluted at high pH from the antibody column suggested that there was little, if any, change in the specific activity of the protein (data not shown).

Comments on the purification scheme. We did not attempt to use ion-exchange chromatography because prolonged exposure of the NS-1 preparations to NaCl concentrations lower than 250 mM caused aggregation and precipitation of the protein. Purified NS-1 preparations also needed to be thawed on ice to keep them active in subsequent assays. If coeluting baculovirus DNA was not removed after the nuclear extraction procedure, lowering of the salt concentration to less than 200 mM NaCl immediately caused the NS-1 to bind to this DNA and subsequent recovery was difficult. Whether this binding is caused by the ADV sequences in the rAcNPV genome or is just nonspecific DNA binding is not known at present. Generally, the purified NS-1 preparations were quite "sticky," but if handled as described, they retained their helicase and ATPase activity for at least 9 months when stored at −80°C.

NS-1 and NS-1K438S ATPase activity. Immunopurified preparations of NS-1 and NS-1K438S were tested for ATPase activity with [^{32}P]ATP as a substrate. The ATPase activity was detected as the release of $^{32}P_i$ after degradation of the radiolabeled ATP into $^{32}P_i$ and ADP. NS-1 and NS-1K438S were tested over a range of concentrations from 25 to 200 ng (Fig. 3), and for wild-type NS-1, ATPase activity was detected even at the lowest concentration and increased as the NS-1 concentration increased. Two hundred nanograms of NS-1 had approximately six times less activity in this assay than 200 ng of baculovirus-expressed SV40 TAg which had been immunoaffinity purified from insect cells. We do not know at present why the ATPase activity was not directly proportional to the concentration of NS-1. Similar observations were made for purified minute virus of mice NS-1 expressed in the insect cell system (53). No ATPase activity was associated with purified NS-1K438S at the concentration range used, indicating that the ATPase activity of the NS-1K438S is at least eight times lower than that of NS-1. This observation also indicated that no ATPase activity was copurifying from the insect cell extracts during the purification procedure, thus confirming that the purified ATPase activity is an intrinsic activity of the NS-1 protein.

The ATPase activity of NS-1 is stimulated by DNA. The level of specific ATPase activity of SV40 TAg is considered to be low compared with that of other ATPases, but its activity can be stimulated up to 10-fold with a variety of DNA polynucleotides (25). Similarly, the nucleotide triphosphatase activities of other helicaselike proteins have been shown to be stimulated by nucleic acids (1, 26, 49). In order to test whether the ATPase activity of purified ADV NS-1 could be stimulated in a similar way, ATPase assays were performed in the presence of 50 to 250 ng of double-stranded (ds) plasmid DNA, ds-poly(dI-dC) or M13 ssDNA (Fig. 4). Under these conditions, the ATPase activity of NS-1 was stimulated up to 5-fold by dsDNA and

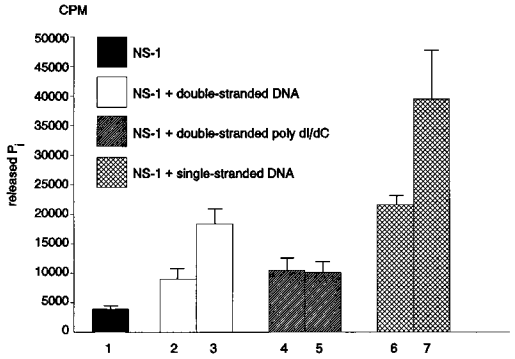

FIG. 4. ATPase activity of NS-1 is stimulated by DNA. A total of 200 ng of NS-1 was incubated under ATPase assay conditions, as described in Materials and Methods, in the presence of the indicated amount of dsDNA or ssDNA. Bars: 1, NS-1 alone; 2, and 3, 50 and 250 ng of dsDNA (pCat-Basic vector; Promega); 4 and 5, 50 and 250 ng of dspoly(dI)-poly(dC) (Pharmacia); 6 and 7, 50 and 250 ng of M13 ssDNA. The specific activity of the ATP was 0.67 mCi/mmol. The assays were performed in triplicate and corrected for the background by subtracting released counts without the presence of NS-1. The values are the means of triplicate determinations represented with 1 standard deviation.

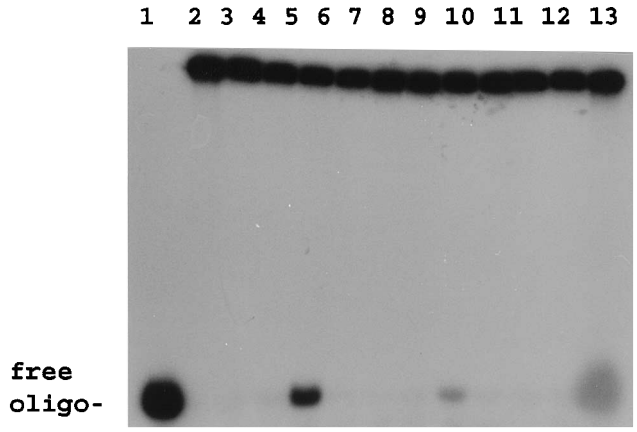

FIG. 5. NS-1 helicase activity: requirement for nucleotide triphosphates. A total of 100 ng of immunopurified NS-1 was incubated in the presence of helicase substrate and different nucleotides as potential energy sources. Lanes: 1, boiled substrate; 2, no NS-1; 3, no $MgCl_2$; 4, no ATP; 5, 4 mM ATP; 6, 4 mM GTP; 7, 4 mM CTP; 8, 4 mM UTP; 9, 4 mM dATP; 10, 4 mM dGTP; 11, 4 mM dCTP; 12, 4 mM dTTP; 13, 100 ng of SV40 TAg.

10-fold by ssDNA. We have also assayed the ATPase activity of NS-1 in the presence of dsDNA containing a part of the ADV genome (nucleotides 239 to 2063), partially dsDNA (the helicase substrate [see below]), or the 26-mer oligonucleotide used for making the helicase substrate. The stimulation observed for dsDNA-containing ADV sequences was similar to that for dsDNA without the ADV sequences, and partially dsDNA in form of the helicase substrate gave essentially the same results as ssDNA alone. The 26-mer single-stranded oligonucleotide was less effective than M13 DNA in enhancing this assay, with 50 ng of the oligonucleotide giving only a 1.5-fold stimulation of the NS-1 ATPase activity.

NS-1 and NS-1K438S helicase activity. It has been reported that the minute virus of mice NS-1 and AAV Rep68 proteins possess helicase activity (29, 53). To discover if the purified fractions of ADV NS-1 exhibited similar activity, immunopurified NS-1 fractions were incubated in the presence of a helicase substrate consisting of a ^{32}P-labeled 26-mer oligonucleotide annealed to M13 ssDNA and with different nucleotides or ribonucleotides serving as energy sources. Helicase activity was detected as displacement of the ^{32}P-labeled oligonucleotide from the M13 ssDNA as assessed by electrophoresis on nondenaturing polyacrylamide gels (Fig. 5). The immunopurified NS-1 fractions had helicase acitivty which was dependent on the presence of Mg^{2+} ions and either ATP or dATP (4 mM) as an energy source, although the level of activity seemed to be a little lower for dATP than for ATP. The nucleotides dGTP, dCTP, and dTTP or the ribonucleotides GTP, CTP, and UTP did not support helicase activity. To investigate the requirements for divalent cations, the helicase assays were carried out in the presence of Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, or Cu^{2+} with ATP as an energy source (Fig. 6). Only Mg^{2+} and Mn^{2+} supported the reaction at all, and the activity seemed to be a little lower in the presence of Mn^{2+} than Mg^{2+}.

To test whether helicase activity could be detected for immunopurified preparations of NS-1K438S, different concentrations (25 to 200 ng) of NS-1K438S were compared with equivalent concentrations of NS-1 in the helicase assay (Fig. 7). No helicase activity was detectable for NS-1K438S, while helicase activity was easily detectable for NS-1. These findings suggested that NS-1K438S has at least an eightfold lower level of helicase activity than wild-type NS-1 and may have no activity at all. Interestingly, no helicase activity was detected for NS-1 in the presence of low concentrations of ATP (0.2 mM), suggesting that the helicase reaction mediated by NS-1 is either very energy demanding or that NS-1 has a low-level affinity for ATP. Alternatively, all of the ATP may be hydrolyzed during the assay incubation time and the released radiolabeled oligonucleotides may simply reanneal.

NS-1 and NS-1K438S ATP binding activity. Because the purified NS-1K438S protein did not show any detectable ATPase or helicase activity, it was of interest to determine whether it was capable of binding ATP. It has been suggested that the invariant lysine residue in the purine binding consensus sequence Gly-X_4-Gly-Lys-Thr of the yeast RAD3 protein contributes to the binding of purine nucleotides by interacting

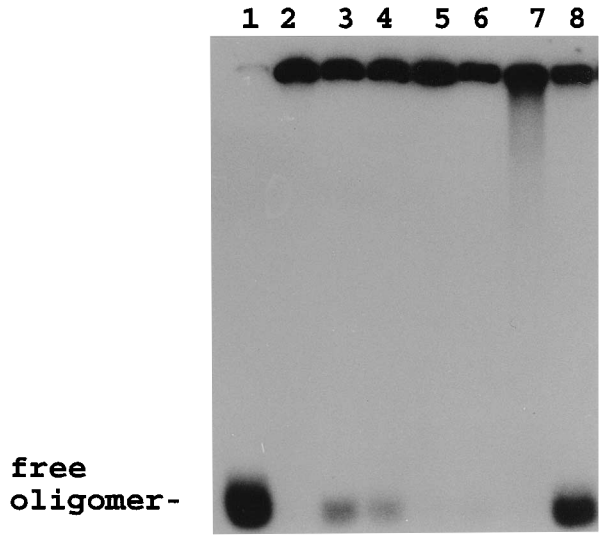

FIG. 6. NS-1 helicase activity: requirement for divalent cations. A total of 100 ng of immunopurified NS-1 was incubated in the presence of helicase substrate, with different divalent cations as cofactors and 4 mM ATP as the energy source. Lanes: 1, boiled substrate; 2, no NS-1; 3, 5 mM $MgCl_2$; 4, 5 mM $MnCl_2$; 5, 5 mM $ZnCl_2$; 6, 5 mM $CaCl_2$; 7, 5 mM $CuSO_4$; 8, 100 ng of SV40 TAg, 5 mM $MgCl_2$, and 4 mM ATP.

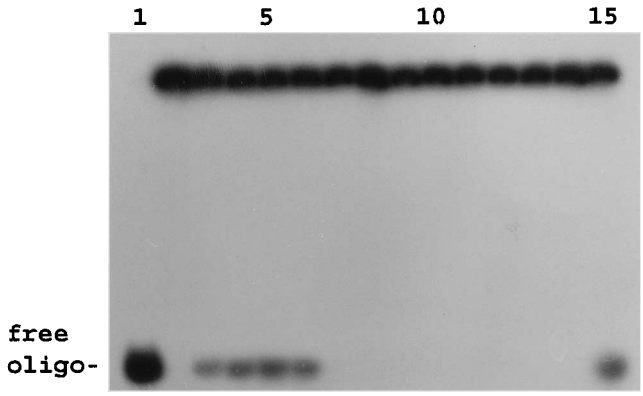

FIG. 7. Comparison of NS-1 and NS-1K438S helicase activity. Different concentrations of NS-1 and NS-1K438S were tested for helicase activity in the presence of 5 mM $MgCl_2$ and 4 or 0.2 mM ATP. Lanes: 1, boiled substrate; 2; no NS-1; 3, 4, 5, and 6, 25, 50, 100, and 200 ng of NS-1, respectively, and 4 mM ATP; 7, 8, 9, and 10, 25, 50, 100, and 200 ng of NS-1K438S, respectively, and 4 mM ATP; 11 and 12, 50 and 200 ng of NS-1, respectively, and 0.2 mM ATP; 13 and 14, 50 and 200 ng of NS-1K438S, respectively, and 0.2 mM ATP; 15, 100 ng of SV40 TAg and 4 mM ATP.

with the alpha and/or beta phosphoryl group(s) of the nucleotide (51). Because ADV NS-1 has a similar consensus sequence, we asked if NS-1K438S retained the ability to bind ATP. ATP binding by NS-1 and NS-1K438S was measured with a gel filtration method adapted from that described by Sung et al. (51). Figure 8 shows the [^{32}P]ATP binding profiles of NS-1 and NS-1K438S with this assay. Both NS-1 and NS-1K438S bound radiolabeled ATP under the assay conditions, although NS-1K438S bound it less well, and in both cases, the binding was efficiently inhibited to background levels with cold ATP. However, the binding of radiolabeled ATP to NS-1K438S, but not to NS-1, could also be inhibited to background levels with cold CTP, indicating that the observed ATP binding of NS-1K438S probably represents nonspecific binding. As seen in Fig. 8, the reduction in binding of [^{32}P]ATP to NS-1K438S in the presence of CTP was the same in absolute terms as the reduction seen for NS-1 with CTP, supporting evidence that all of the ATP binding of NS-1K438S is of a nonspecific

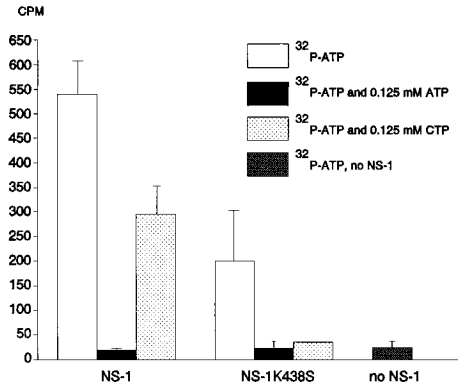

FIG. 8. Comparison of ATP binding of NS-1 and NS-1K438S. NS-1 and NS-1K438S were incubated in the presence of [^{32}P]ATP at 4°C. Protein-bound [^{32}P]ATP was separated from free [^{32}P]ATP by filtration of the reaction mixtures through Sephadex G-50 columns. The specificity of the assay was evaluated with either unlabeled 0.125 mM ATP or 0.125 mM CTP as the competitor for [^{32}P]ATP binding. The values are the means of triplicate determinations represented with 1 standard deviation. (The NS-1K438S assay incubated in the presence of [^{32}P]ATP and 0.125 mM CTP was done in duplicate.)

nature. Altogether, these data indicate that NS-1K438S has at least 10- to 15-fold lower specific affinity for ATP than NS-1.

DISCUSSION

The major nonstructural proteins of parvoviruses are absolutely required for viral replication both in vivo and in vitro (15, 47). These proteins bind directly to specific sequence elements in the replication origins of the viral genomes (14, 36, 37) and may, like the SV40 TAg, somehow recruit and coordinate the cellular replication machinery during viral DNA synthesis (52). The NS-1 proteins of the autonomous parvoviruses are also potent transactivators of parvovirus transcription and have been shown to bind directly to a critical region in the minute virus of mice P38 promoter, termed the transactivating region (tar) (10, 48). In contrast, NS-1 down-regulates transcription from a number of cellular and viral promoters (48). The development of in vitro transcription and replication assays and the identification of cellular factors involved in parvovirus transcription and replication have both been hampered by the lack of pure, active preparations of NS-1. In this paper, we have described the expression and purification of ADV NS-1 in insect cells, in the hope that the purified reagent may facilitate such studies. The purification scheme consisted of a nucleus salt extraction procedure, immobilized Zn^{2+} ion chromatography, and a final immunoaffinity chromatography step. The yields of purified NS-1 and NS-1K438S were in the range of 0.5 to 10 mg/10^9 cells, which is approximately 20 to 400 times the amount expressed during ADV infection in cell culture. The NS-1 fractions eluted from the immunoaffinity column were highly purified and exhibited ATPase activity and Mg^{2+}/Mn^{2+} ATP/dATP-dependent helicase activity. The activities observed were compared with those of SV40 TAg purified from an insect cell source, and the specific activities observed for the NS-1 preparation were found to be approximately three times lower. It was difficult to assess whether or not NS-1 lost specific activity during the purification procedure, because the helicase and ATPase assays also detected the activity of cellular contaminants. However, the NS-1 preparations were already highly purified after the Zn^{2+} ion chromatography step and we did not observe any significant loss of helicase activity when these preparations were taken through the immunoaffinity step (data not shown). It should be noted that NS-1K438S preparations, which had only been purified by Zn^{2+} ion chromatography, showed no detectable helicase activity, indicating that by this stage in the purification endogenous helicases from the baculovirus-infected insect cells had been eliminated. If very high levels of purity of NS-1 are not required in a given assay, the Zn^{2+} ion chromatography eluate might be a good source of NS-1. Whether the specific intrinsic activities like ATPase, helicase, putative endonuclease, and DNA binding activities, etc., of the NS-1 preparations are similar to those of NS-1 expressed during ADV infection in cell culture and are able to function in in vitro replication/transcription assays cannot be answered, because these assays have not yet been developed for ADV. However, a good indication that the preparations may work is that the SV40 TAg preparation used for comparison was highly active in SV40 in vitro replication (data not shown), and recently baculovirus-expressed Rep proteins and minute virus of mice NS-1 have been demonstrated to be active in in vitro replication assays (35, 38). It might be of functional importance, for putative posttranslational modifications such as phosphorylation, etc., that ADV NS-1 was found to be localized to the nuclei of the Sf9 cells, in contrast to baculovirus-expressed minute virus of mice NS-1, which has been described to be cytoplasmic (53).

Like other helicases (1, 25, 26, 49), the ATPase activity of ADV NS-1 was found to be stimulated by ssDNA and, to a lesser extent, by dsDNA. The functional significance of this observation is, at the moment, not clear. However, when considering the ATPase activity of NS-1 along with its known role in binding to specific sequences in the parvovirus genome, including the replication origins (10, 14), that role involves NS-1 binding of ATP, probably followed by a dimerization/oligomerization of the peptide (10, 14, 41), which seems to be an obligatory event before binding the specific recognition sequence(s), for example, in the replication origin(s) (10, 14). The low level of specific ATPase activity of NS-1 might stabilize oligomeric forms of NS-1. The dsDNA at the origin might then stimulate the ATPase activity of NS-1, presumably resulting in local unwinding catalyzed by the intrinsic helicase activity of NS-1 (30, 53). Subsequently, NS-1 nicks at the nick site recognition sequence and becomes covalently attached on one of the generated DNA single strands by using the intrinsic endonuclease activity in NS-1 (16, 29, 39); at the same time, the hydrolysis of ATP could be accelerated by the locally generated DNA single strands, causing NS-1 to lose affinity to dsDNA and dissociate or bind to the generated ssDNA to stabilize the subsequent formation of the replication initiation complex.

It has been reported that terminal resolution reactions supported by the purified AAV Rep78 and Rep68 proteins show somewhat different nucleotide requirements. Reactions mediated by Rep78 required ATP, but similar reactions with Rep68 could be demonstrated in the presence of ATP, CTP, GTP, or UTP, and only Mg^{2+} supported the full reaction for both proteins (29, 30). When the nucleotide and divalent cation requirements of immunopurified ADV NS-1 preparations were tested in the helicase assay, we found that these reactions depended on the presence of ATP or dATP and Mg^{2+} or Mn^{2+} but that GTP, CTP, and UTP were not able to support helicase activity. These observations suggest that there are differences in the substrate and cofactor requirements of AAV Rep proteins and ADV NS-1. However, the trs assay is more complex than the helicase assay. It consists of a DNA binding and unwinding step, followed by introduction of a site-specific nick and covalent attachment to the template DNA (29). It has been proposed that the nicking reaction mediated by Rep or NS-1 requires a divalent cation coordinating the amino acid sequence HuHuuu (where H is histidine and u is a bulky hydrophobic residue). This motif is conserved among the replicator proteins of many systems which use rolling-circle DNA replication mechanisms (28). Thus, the finding that Mn^{2+} functions as cofactor in the helicase assay but not in the trs assay may mean that Mn^{2+} can support the helicase activity of both proteins but the nicking and covalent attachment reaction is exclusively catalyzed by Mg^{2+} coordinated by the consensus sequence mentioned above.

Mutations in the consensus purine binding site of H-1 and minute virus of mice NS-1 polypeptides in which the critical lysine residue was replaced by serine or methionine and arginine, respectively, abolished all of the proteins' known biological functions, such as replication in vivo and in vitro, transactivation, and cytotoxicity (34, 40). We have shown earlier that the ADV NS-1K438S mutation destroys the proteins' transactivation potential but that this mutant does not inhibit transactivation by wild-type NS-1 when expressed in the same cell, and we suggested that ATP binding might be necessary for protein oligomerization, DNA binding, and transactivation (12). In this article, we have presented evidence that the serine mutation severely decreases the intrinsic ATPase and helicase activities of the polypeptide and have shown that NS-1K438S is not able to bind ATP. For this, we used an ATP binding assay as previously described for the yeast RAD3 protein, which, like the ADV NS-1 polypeptide, has a type A consensus nucleotide binding sequence and possesses helicase and ssDNA-dependent ATPase activity. According to the data obtained for the RAD3 protein, replacing the invariant positively charged lysine with a serine which possesses a negative polar charge would be expected to render NS-1K438S incapable of hydrolyzing or even binding ATP (51). Moreover, it has been shown that replacing the equivalent invariant lysine in the Semliki Forest virus nsP2 helicase with asparagine causes inactivation of ATPase activity, while a similar substitution in the human Ha-ras-encoded protein causes a 100-fold reduction in the affinity of the protein for GTP (49, 50). The ATP binding data we obtained confirmed that NS-1K438S had a much lower level of specific affinity for ATP than wild-type NS-1, and we could not detect any ATPase activity in four different preparations of NS-1K438S even if assayed after the Zn^{2+} ion chromatography step. Thus, our results conflict with recently published findings on the corresponding NS-1 mutant of minute virus of mice (NS-1K405S): the ATPase activity for this mutant was only found to be reduced 13% compared with that of wild-type minute virus of mice NS-1 (31). One possible explanation could be that the ATPase activity measured for the preparations of minute virus of mice NS-1K405S represented contaminating cellular ATPase activity, because these preparations were estimated to be only 85% pure. This explanation is supported by the observation that minute virus of mice NS-1 molecules with methionine or arginine substitutions at amino acid 405 are unable to oligomerize (41) and that the specific DNA binding activity of these mutants is not stimulated by ATP as is found for wild-type minute virus of mice NS-1 (14), indicating that these mutants interact abnormally with ATP. Alternatively, the lysine-to-serine substitution at this position may simply influence the ATPase activity of the ADV and minute virus of mice NS-1 proteins differently.

Interestingly, NS-1K438S was expressed at 5- to 10-fold higher levels than wild-type NS-1, probably because it was less cytotoxic to the cell. Moreover, NS-1K438S could be produced in milligram quantities relatively easily and can therefore be an important source of protein for vaccine trials and diagnosis of ADV infection. Such studies are in progress. This mutant could also be an important source of NS-1 for three-dimensional structural studies, both because of its availability and, perhaps more importantly, because this mutation might inhibit oligomer formation and conformational changes in the polypeptide, thus giving more homogeneous preparations.

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