Partial Purification of Adeno-Associated Virus Rep78, Rep52, and Rep40 and Their Biochemical Characterization

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We have used differential cell extraction and conventional chromatography to separate and partially purify the four adeno-associated virus (AAV) nonstructural proteins Rep78, Rep68, Rep52, and Rep40. In the cytoplasmic extracts Rep52 and Rep40 were present in greater abundance than Rep68 and Rep78, with Rep78 being the least abundant. In nuclear extracts the four Rep proteins were approximately equal in abundance. Regardless of the subcellular fraction examined, three of the Rep proteins (Rep78, Rep68, and Rep40) consisted of two protein species with slightly different mobilities during polyacrylamide gel electrophoresis. In contrast, Rep52 consisted of only one protein species. Both Rep78 and Rep68 were capable of binding efficiently to AAV terminal hairpin DNA substrates, but we could not detect site-specific DNA binding by Rep52 and Rep40. Like Rep68, Rep78 had both an ATP-dependent *trs* endonuclease and a DNA helicase activity. Both Rep78 and Rep68 cut the terminal AAV sequence at the same site (nucleotide 124). The binding, *trs* endonuclease, and DNA helicase activities comigrated during sucrose density gradient centrifugation with a mobility expected for a monomer of the protein, suggesting that the three biochemical activities were intrinsic properties of the larger Rep proteins. The chromatographic behavior and the DNA-binding properties of the four Rep proteins identified at least two domains within the *rep* coding region, an exposed hydrophobic domain within the C-terminal end (amino acids 578 to 621) and a region within the N terminus (amino acids 1 to 214) which was necessary for binding to the terminal repeat sequence. No site-specific nuclease activity was seen in the presence of nucleotide analogs ATP- γ -S or AMP-PNP, suggesting that ATP hydrolysis was required for the endonuclease reaction. Furthermore, although ATP was the only cofactor which would support the *trs* endonuclease activity of Rep78, Rep68 nuclease activity was seen in the presence of several other nucleotide cofactors, including CTP, GTP, and UTP.

Adeno-associated virus (AAV) is a linear, single-stranded, human DNA virus (3) which requires coinfection with herpes simplex virus or adenovirus for efficient AAV DNA replication (3, 5, 27). The helper virus gene products are required to facilitate AAV gene expression but do not appear to be directly involved in AAV DNA replication (16, 31, 36, 43). Genetic studies indicate that two AAV genes are required for viral DNA replication, the inverted terminal repeats and the *rep* coding region (13, 30, 32, 39). The palindromic terminal repeat forms a hairpinned, T-shaped secondary structure which is used as a primer for the synthesis of a viral replicative-form DNA in which one end is covalently joined in a hairpinned configuration (1, 4, 7, 21-23, 37). The hairpin end is resolved by a site-specific, strand-specific nick at a position near the end of the terminal palindrome called the terminal resolution site (*trs*) (4, 15, 33, 34).

The *rep* gene codes for a family of at least four nonstructural proteins, Rep78, Rep68, Rep52, and Rep40 (Fig. 1A) (2, 6, 11, 12, 20, 25, 35, 41, 42). The two larger Rep proteins, Rep78 and Rep68, are synthesized from unspliced and spliced transcripts initiated at the p5 promoter; the smaller Rep proteins, Rep52 and Rep40, are synthesized from the p19 transcripts (Fig. 1A). Mutations in the p5 Rep proteins are defective for viral DNA replication (13, 39) and for transactivation of viral gene expression (18, 40) and are at least partially defective for repression of heterologous genes (17).

In previous work we developed in vitro biochemical assays in which various aspects of the terminal resolution

reaction could be studied (14, 15, 33, 34). These studies suggested first that either Rep68 or Rep78 (or both) was capable of binding tightly to the AAV terminal repeat sequence when it was in the hairpin configuration (14). We also purified one of the four Rep proteins, Rep68, to apparent homogeneity and showed that it had a number of biochemical activities that are related to the terminal resolution reaction (15, 32, 33). First, Rep68 was an ATP-dependent, site-specific, and strand-specific endonuclease which cuts at the AAV *trs* site (15, 33). Second, during the *trs* endonuclease reaction (Fig. 1C), Rep68 became attached to the 5' end of the nick via a covalent tyrosine-phosphate linkage (15, 33, 34). Third, Rep68 contained a tightly associated DNA helicase activity (Fig. 1B) (15). Finally, both biochemical and genetic studies suggested that the p19 Rep proteins did not have a role in terminal resolution (9, 32).

In this report we continue our studies of the AAV Rep proteins. First, we describe a method for the separation and partial purification of the other three AAV Rep proteins, Rep78, Rep52, and Rep40. Second, we report that, with the exception of its nucleotide cofactor requirement, Rep78 was found to have *trs* endonuclease, terminal repeat binding, and DNA helicase activities similar to those of Rep68. We also provide additional evidence that the *trs* endonuclease and DNA helicase activities are intrinsic to the p5 Rep proteins, and we demonstrate that hydrolysis of ATP is required for the *trs* endonuclease reaction. Finally, we present evidence that the p19 Rep proteins bind poorly to the AAV terminal repeats, suggesting that amino acids 1 to 214 of the Rep78 (or Rep68) protein contain at least one region of the protein that is necessary for binding the AAV terminal repeats.

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FIG. 1. (A) AAV *rep* gene. The diagram of the AAV genome shows the terminal repeats (solid boxes), the *rep* open reading frame (open box), and the positions of the two promoters, p5 and p19 (bent arrows), which initiate the synthesis of the unspliced and spliced mRNAs (see below) used for the synthesis of the four AAV Rep proteins. The numbers in parentheses indicate the theoretical molecular masses in kilodaltons of Rep78, Rep68, Rep52, and Rep40, respectively. (B) DNA helicase assay. The substrate for the assay consists of a 3' 32 P-labeled 26-base oligonucleotide annealed to M13 ssDNA. Displacement of the oligonucleotide after incubation with the Rep protein is detected by electrophoresis of the reaction products on a nondenaturing acrylamide gel. (C) *trs* endonuclease assay. The substrate for the assay is the hairpinned terminal *Xba*I fragment, isolated from NE DNA and 32 P-labeled at its unique 5' end (asterisk). When incubated with Rep68, the substrate is nicked at the terminal resolution site (*trs*) and the 5' end of the nick becomes covalently attached to the Rep protein through a tyrosine residue (15, 33, 34). Some of the product also undergoes DNA helicase activity to produce a single-stranded 73-base fragment and a reciprocal 192-base fragment which is still attached to the Rep protein (15, 33). Incubation of the initial reaction products at 100°C denatures all of the nicked substrate to produce the 32 P-labeled 73-base fragment and the unlabeled 192-base PDC. The same 5'-labeled *Xba*I substrate is used for the DNA-binding gel shift assay.

MATERIALS AND METHODS

Nucleotides and nucleic acids. Unlabeled deoxy- and ribonucleoside triphosphates were purchased from Sigma or Pharmacia; [γ - 32 P]dATP and [α - 32 P]dCTP were from ICN. Salmon sperm DNA was purchased from Sigma and treated with phenol and chloroform, precipitated with ethanol, and dissolved in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA (TE). Poly (dI-dC) was purchased from Pharmacia.

Gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the method of Laemmli (19). Gel electrophoresis with nondenaturing 6% polyacrylamide or denaturing 6% acrylamide–8 M urea gels was done by using Tris-borate-EDTA buffer (pH 8.3) as previously described (14, 15). Protein high-molecular-weight markers were purchased from Bio-Rad; prestained protein markers were from Bethesda Research Laboratories.

Substrate for *trs* endonuclease and DNA-binding assays. AAV no-end DNA (NE DNA) was enzymatically prepared from *psub201(+)* plasmid DNA as described previously (29, 34). NE DNA has two identical hairpinned termini bounded by *Xba*I sites. To obtain the 5'-end-labeled DNA termini, NE DNA was digested with *Xba*I and the *Xba*I ends were

treated with calf intestinal phosphatase (CIP) and T4 polynucleotide kinase in the presence of [γ - 32 P]ATP. The 5'-end-labeled *Xba*I fragment (265 nucleotides) was then purified by gel electrophoresis and electroelution. The hairpinned *Xba*I fragment was the substrate for both the *trs* endonuclease and DNA-binding assays (see below) (Fig. 1C).

DNA helicase substrate. A 17-base oligonucleotide (universal primer; United States Biochemical Corp.) was annealed to 1 μ g of single-stranded M13mp18 DNA under the conditions used for DNA sequencing. The primer was then extended by using T7 DNA polymerase (Sequenase; United States Biochemical Corp.) in the presence of 0.75 mM each dGTP, dTTP, and [α - 32 P]dATP at 37°C for 30 min. Unlabeled dATP was then added to the reaction mixture to a final concentration of 0.75 mM, and the reaction was incubated at 37°C for an additional 15 min. Unincorporated nucleotides and primers were removed by Bio-Gel 5M gel filtration (Bio-Rad). This procedure labeled the primer at its 3' end and extended the primer to a final length of 26 bases (Fig. 1B).

DNA-binding gel shift assay. The gel shift assay was performed as previously described (14). Briefly, the reaction mixture (20 μ l) contained 25 mM HEPES(*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 2% glycerol, 12.5 μ g of bovine serum albumin (BSA) per ml, 50 mM NaCl, 0.01% Nonidet P-40, 50 μ g of either poly(dI-dC) or sonicated salmon sperm DNA per ml, 5 ng of 32 P-labeled NE *Xba*I fragment per ml, and 1 to 5 μ l of the protein solution.

***trs* endonuclease assay.** The *trs* endonuclease assay (Fig. 1C) contained, in a reaction mixture of 20 μ l; 25 mM HEPES-KOH (pH 7.5), 5 mM MgCl₂, 1 mM dithiothreitol, 0.4 mM ATP, 10 μ g of BSA per ml, 25 ng of 32 P-labeled NE *Xba*I fragment per ml, and 1 to 5 μ l of protein solution. The reaction was incubated for 60 min at 37°C and stopped by the addition of 10 μ l of gel-loading buffer (0.5% SDS, 50 mM EDTA [pH 7.5], 40% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol). The products of the reaction were separated by gel electrophoresis. Following electrophoresis the gels were dried and autoradiographed, and in some cases the radioactivity in each band was counted by a scanning gas flow counter (Ambis, Inc.). If the products were to be separated on a 6% acrylamide–8 M urea DNA sequencing gel, the reaction mixture was treated with proteinase K (0.5 mg/ml at 37°C for 1 h), extracted with phenol and chloroform, and precipitated with ethanol prior to electrophoresis. If the total amount of nicked substrate was to be determined, the reaction products were heated at 100°C for 5 min prior to electrophoresis on a 6% nondenaturing polyacrylamide gel (Fig. 1C).

DNA helicase assay. The helicase assay (Fig. 1B) conditions were identical to those described above for the *trs* endonuclease assay, except that 50 μ g of 32 P-labeled M13mp18 helicase substrate per ml was used. The reaction mixture was incubated for 30 min at 37°C, and then the reaction was stopped by the addition of 10 μ l of gel-loading buffer. The reaction mixture was then electrophoresed on a nondenaturing 6% polyacrylamide gel.

Immunoblotting assay. Immunoblotting assays were performed as previously described (14, 15), except that the anti-peptide rabbit polyclonal antibody was used at a 1:1,000 dilution. Whole-cell extracts were prepared as described previously (14) from HeLa cells infected with adenovirus and AAV. When testing individual column fractions for the presence of Rep proteins, it was often necessary to concentrate the protein before gel electrophoresis. Typically 90 μ l

FIG. 2. (A) Cell fractionation. The diagram illustrates the procedure used to fractionate cells infected with adenovirus (Ad) and AAV to produce the subcellular S-100 extracts used in this study for the purification of the Rep proteins (see Materials and Methods for details). (B) Rep purification. The diagram illustrates the chromatographic steps used to fractionate the 1 M S-100 nuclear extract to separate the four Rep proteins (Rep78, Rep68, Rep52, and Rep40). A similar scheme was used to fractionate the cytoplasmic and 0.2 M nuclear extracts (see Materials and Methods for details).

of every other fraction was precipitated with 400 μ l of acetone in the presence of 10 μ l of 10% SDS. The precipitate was dissolved in 40 μ l of Laemmli sample buffer (19) and loaded onto an SDS-8% polyacrylamide gel.

Preparation of cytoplasmic and nuclear extracts. Eight liters of HeLa suspension cells was grown at 37°C in Eagle's minimal essential medium supplemented with 5% calf serum, 1% glutamine, penicillin, and streptomycin. At a density of 5×10^5 cells per ml the cells were infected with adenovirus type 2 (multiplicity of infection = 10) and AAV2 (multiplicity of infection = 20). The cells were harvested approximately 30 h after infection. Nuclear and cytoplasmic fractions were prepared as described by Chalberg and Kelly (8) and frozen at -70°C. The cytoplasmic extract was centrifuged at $100,000 \times g$ for 60 min prior to fractionation. To prepare the 0.2 M NaCl nuclear extract (Fig. 2A), the frozen nuclei were thawed and resuspended in 30 ml of buffer A (25mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.01% Nonidet P-40). The nuclear suspension was adjusted to 0.2 M NaCl and extracted for 1 h on ice. The nuclear suspension was then centrifuged at $100,000 \times g$ for 60 min, and the clear supernatant was called the 0.2 M S-100 extract. To prepare the 1 M NaCl nuclear extract (Fig. 2A), the pellet was resuspended in buffer A containing 1 M NaCl [or 1 M $(\text{NH}_4)_2\text{SO}_4$] and extracted for 1 h on ice. The 1 M nuclear suspension was then centrifuged at $100,000 \times g$ for 60 min, and the clear supernatant was called the 1 M S-100 extract.

Purification of Rep fractions. The purification of the

Rep68(I) fraction from 0.2 M extracts has been described previously (15). The other Rep proteins were purified from all three types of extracts (cytoplasmic, 0.2 M nuclear, and 1 M nuclear) essentially by the same procedure (Fig. 2B). Each extract was adjusted to 1 M NaCl and applied to a phenyl-Sepharose CL-4B (Pharmacia) column (bed volume, 4 ml; diameter 1.6 cm) that had been equilibrated with buffer B [buffer A containing 1 M $(\text{NH}_4)_2\text{SO}_4$]. The column was washed with 5 column volumes of buffer B and eluted with a descending linear gradient (10 column volumes) of $(\text{NH}_4)_2\text{SO}_4$ (1 to 0 M) in buffer A. The phenyl-Sepharose fractions which contained Rep proteins were identified by immunoblotting and gel shift assays. Two major peaks of Rep protein were usually found. One was enriched for the spliced Rep proteins (Rep68 and Rep40); the other was enriched for the unspliced Rep proteins (Rep78 and Rep52). The active phenyl-Sepharose fractions were pooled and dialyzed against buffer C (buffer A containing 50 mM NaCl and 20% glycerol). Each phenyl-Sepharose pool was then loaded onto a separate DEAE-52-cellulose (Whatman) column (bed volume 2 to 4 ml; diameter, 1.2 cm) that had been equilibrated with buffer C. The Rep proteins (Rep52 and Rep40) which did not bind to the DEAE columns (DEAE flowthrough fractions) were saved and stored at -70°C. The DEAE columns were washed with 5 column volumes of buffer C and eluted with a linear gradient (10 column volumes) of NaCl (50 to 600 mM) in buffer C. Fractions which contained Rep proteins were identified by the DNA-binding assay and by immunoblotting. The active fractions were pooled and dialyzed against buffer C (DEAE-bound fractions). The pooled DEAE fractions were then applied to single-stranded DNA (ssDNA)-agarose columns (wet volume, 0.5 to 1 mg of DNA per ml; bed volume, 1 to 4 ml; diameter, 1.2 cm; Bethesda Research Laboratories) that had been equilibrated with buffer C, and the column was washed with 5 column volumes of loading buffer. The flowthrough fraction of the ssDNA-agarose column that had been loaded with the DEAE Rep68 pool contained significant amounts of Rep68 and was called Rep68(II). The ssDNA columns were subsequently eluted with linear gradients (10 column volumes) of NaCl (0.05 to 1 M) in buffer C. Fractions that contained Rep68 (or Rep78) were identified by immunoblotting and by the DNA-binding, *trs* endonuclease, and DNA helicase assays. Active fractions were pooled, dialyzed against buffer C, and concentrated by Amicon Centriflo 25 centrifugation. The pooled ssDNA-agarose fractions were called Rep68(I) and Rep78.

Sucrose gradient centrifugation. Rep68(I) (200 μ l) was dialyzed against sucrose gradient buffer (50 mM Tris · HCl [pH 7.5], 5 mM MgCl_2 , 0.5 mM dithiothreitol, 50 mM NaCl, 0.1 mM EDTA, 0.01% Nonidet P-40) and applied to a 4.8-ml sucrose gradient (10 to 30%). The centrifugation was performed in an SW65 Beckman rotor at 40,000 rpm for 24 h at 4°C. After centrifugation, 150- μ l fractions were taken manually from the top of the gradient. Every other fraction was assayed for DNA-binding, *trs* endonuclease, and DNA helicase activities.

RESULTS

Differential extraction of AAV-infected cells. Because previous reports (25, 42) had suggested that the p5 Rep proteins were more abundant in the nucleus, we fractionated HeLa cells that had been infected with adenovirus and AAV into cytoplasmic and nuclear fractions. We then extracted the infected nuclei sequentially with 0.2 and 1.0 M NaCl (Fig.

FIG. 3. (A) Relative abundance of the Rep proteins in subcellular fractions. Aliquots of a whole-cell extract (WCE), the cytoplasmic extract (Cytosol), and the 0.2 M and 1.0 M nuclear extracts (Nu) were electrophoresed on an SDS-8% polyacrylamide gel and immunoblotted with Rep-specific polyclonal antibody. Whole-cell extracts from cells that were uninfected (UNI) or infected only with adenovirus (Ad) are also shown. Numbers between panels A and B identify the Rep protein species. (B) Immunoblotting analysis of phenyl-Sepharose fractions. The pooled fractions which contained the peaks of spliced (Rep68 plus Rep40 [68 + 40]), and unspliced (Rep78 plus Rep52 [78 + 52]) Rep protein during phenyl-Sepharose chromatography of the cytoplasmic and 0.2 M and 1 M nuclear S-100 extracts are shown. (C) DEAE-cellulose chromatography of the spliced and unspliced phenyl-Sepharose Rep protein fractions obtained from the 1 M nuclear extract. The Rep78-plus-Rep52 (78 + 52) and Rep68-plus-Rep40 (68 + 40) phenyl-Sepharose (PS) fractions were fractionated on separate DEAE-cellulose columns. In each case the p5 Rep protein (Rep78 or Rep68) was found in the DEAE-cellulose-bound (DE Bnd) fraction while the p19 Rep protein (Rep52 or Rep40) was found in the DEAE cellulose flowthrough fraction (DE FT). Immunoblotting conditions were as described for panel A. Other abbreviations: WCE, adenovirus-plus-AAV-infected whole-cell extract; 1M Nu Ext, 1 M nuclear S-100 extract.

2A). The three fractions (cytoplasmic, 0.2 M nuclear, and 1.0 M nuclear) were examined by immunoblotting to determine the relative abundance of the four Rep proteins in each fraction (Fig. 3A). The cytoplasmic extract was found to contain relatively more of the p19 Rep proteins (Rep52 and Rep40) than the p5 Rep proteins when compared with the nuclear extracts. Rep78 was particularly underrepresented in the cytoplasmic extract (Fig. 3A), and we were unable to detect any Rep78 in most other cytoplasmic preparations (33; data not shown). The two nuclear extracts contained Rep78, Rep68, and Rep52 in approximately equal amounts. Rep40 was the most abundant protein in all three extracts (Fig. 3A). It was noted previously that some of the Rep proteins were heterogeneous in molecular weight (14, 15,

28). In this study, three of the Rep proteins (Rep78, Rep68, and Rep40) could be resolved by acrylamide gel electrophoresis as two species of slightly different molecular weight (Fig. 3); only one species of Rep52 was seen. All three extracts, the cytoplasmic and the 0.2 M and 1.0 M NaCl nuclear extracts, were fractionated as described below in an attempt to separate the four Rep proteins.

Separation of the four Rep proteins by phenyl-Sepharose and DEAE-cellulose chromatography. In principle, the four Rep proteins could be resolved on the basis of the differences in their amino acid composition. The two p19 Rep proteins (Rep52 and Rep40) were missing amino acids 1 to 214 of the N terminus of the *rep* open reading frame, and the two spliced Rep proteins (Rep68 and Rep40) were missing the amino acids encoded by the intron (amino acids 578 to 621) in the C-terminal end of the *rep* open reading frame. The C-terminal intron sequence contained a substantial stretch of hydrophobic amino acids, and we reasoned that if the intron-encoded amino acids were part of an exposed surface domain, it should be possible to separate the spliced (Rep68 and Rep40) and unspliced (Rep78 and Rep52) Rep proteins by hydrophobic interaction chromatography. This proved to be correct. The protein elution profiles during phenyl-Sepharose chromatography were essentially the same in the cytoplasmic and 0.2 M and 1 M nuclear extracts (data not shown). Rep68 and Rep40, the spliced Rep products, eluted first as a single peak between 800 and 400 mM $(\text{NH}_4)_2\text{SO}_4$. Rep78 and Rep52 eluted between 400 and 0 mM $(\text{NH}_4)_2\text{SO}_4$, with Rep52 generally eluting after Rep78.

The purity of the spliced and unspliced Rep fractions obtained from the cytoplasmic and 0.2 M and 1.0 M nuclear extracts was examined by immunoblotting (Fig. 3B). All of the pooled Rep68-plus-Rep40 phenyl-Sepharose fractions were free of contamination by Rep78 or Rep52. The converse, however, was not always true. The Rep78-plus-Rep52 phenyl-Sepharose pools from the cytoplasmic and 0.2 M nuclear extracts contained significant amounts of contaminating Rep68 and Rep40 (Fig. 3B). Only for the 1.0 M nuclear extract was it possible to cleanly separate Rep78 and Rep52 from Rep68 and Rep40 with little detectable cross-contamination. We therefore focused on fractionating the 1.0 M phenyl-Sepharose pools further to complete the separation of the four Rep proteins. (Although the cytoplasmic and 0.2 M nuclear extracts were not a good source for the isolation of a pure Rep78-plus-Rep52 fraction, they were a good source of Rep68-plus-Rep40, and we have described previously the purification of Rep68 from the 0.2 M nuclear and cytoplasmic extracts [15].)

Rep78 was separated from Rep52 and Rep68 from Rep40 by DEAE-cellulose chromatography of the Rep78-plus-Rep52 and Rep68-plus-Rep40 phenyl-Sepharose fractions (Fig. 2B and 3C). Rep52 and Rep40 did not bind to DEAE-cellulose in a protein-loading buffer containing 40 mM NaCl and were identified by immunoblotting in the DEAE flowthrough fractions (Fig. 3C). Rep78 and Rep68, which did bind to DEAE-cellulose, were eluted with salt. The four fractions obtained by DEAE chromatography each contained one of the Rep proteins, and each was essentially free of contamination by the remaining Rep proteins (Fig. 3C).

To compare the biochemical activities of the two p5 Rep proteins in more detail, it was necessary to remove a number of contaminating enzymes that were likely to inhibit the *trs* endonuclease or DNA helicase activities of the Rep proteins. Our previous experience with the purification of Rep68 from the cytoplasmic and 0.2 M nuclear extracts (15) suggested that ssDNA-agarose chromatography was a suitable step.

Therefore, the DEAE pools of Rep78 and Rep68 which were obtained from the 1.0 M nuclear extracts were purified further by ssDNA-agarose chromatography. Attempts to purify the DEAE Rep52 and Rep40 fractions further have been unsuccessful thus far. Neither of the p19 Rep proteins was capable of binding to carboxymethyl cellulose or ssDNA-agarose. Because no biochemical activity was detected in the Rep52 and Rep40 fractions (see below), it was difficult to estimate the extent of their purification. A comparison of the immunoblots suggested that neither Rep52 nor Rep40 were purified more than 10-fold over the starting material. The extent of purification of Rep78 and Rep68 was also difficult to calculate because the *trs* endonuclease assay was unreliable in measuring activity in crude extracts and because the total *trs* endonuclease activity was split during purification among several chromatographically distinct fractions (see below). We estimated that the Rep68(I) and Rep78 ssDNA agarose fractions described below were both purified approximately 200- to 1,000-fold over the starting material.

Two chromatographically distinct forms of Rep68. When the DEAE Rep68 fraction was loaded onto ssDNA-agarose, two chromatographically distinct forms of Rep68 were isolated (Fig. 2B). A portion of the Rep68 was incapable of binding to ssDNA and was detected by immunoblotting in the flowthrough (unbound) fraction (data not shown). The fraction which bound to ssDNA-agarose was called Rep68(I), and the flowthrough fraction was called Rep68(II). This phenomenon was not due to overloading of the column beyond its protein-binding capacity, because an increase in the column volume did not significantly affect the amount of Rep68 found in the flowthrough fraction. Furthermore, if the Rep68 in the flowthrough fraction was reloaded onto a new ssDNA-agarose column, it did not result in any significant additional binding of Rep68. The two forms of Rep68 were also seen when the DEAE Rep68 fractions that had been isolated from cytoplasmic and 0.2 M nuclear extracts were loaded onto ssDNA-agarose. In contrast to the chromatographic behavior of Rep68 on ssDNA-agarose, we could not detect any Rep78 in the flowthrough fraction during ssDNA-agarose chromatography (Fig. 2B); all of the Rep78 present in the DEAE fraction bound to ssDNA.

ssDNA-agarose elution profiles of Rep68(I) and Rep78. The ssDNA-agarose elution profiles of Rep68(I) and Rep78 were compared by immunoblotting and by the use of the DNA-binding (gel shift) assay, the M13 DNA helicase assay, and the *trs* endonuclease assay (Fig. 4 and 5). Two types of *trs* endonuclease reactions were performed (Fig. 1C). In one type the products of the reaction were heat denatured to determine the total amount of starting substrate that had been cut during the reaction (Fig. 4B and 5B, circles). In the other type the products of the endonuclease reaction were not denatured prior to electrophoresis, so as to visualize the types of protein-DNA complexes that the two Rep proteins were capable of forming (Fig. 4A and 5A).

Both Rep68 and Rep78 were judged by immunoblotting to be essentially free (>95%) of each other (Fig. 4C and 5C). In addition, Rep68 and Rep78 were each found to exist as two protein species with slightly different mobilities during SDS-polyacrylamide gel electrophoresis. The peak fractions of Rep78 were capable of forming a higher-molecular-mass complex of Rep78 (Fig. 5C, fractions 13 to 16) which was stable during SDS-polyacrylamide gel electrophoresis. The apparent molecular mass of this complex, 140 kDa, was consistent with its being a homodimer of Rep78.

The peak of Rep68 protein (Fig. 4C, fractions 6 to 10)

FIG. 4. (A) ssDNA-agarose chromatography of the Rep68 DEAE fraction from a 1 M nuclear extract. Aliquots (3 μ l) of the indicated fractions were assayed under standard *trs* endonuclease reaction conditions. After incubation, the reaction products without heat denaturation were electrophoresed on a 6% nondenaturing polyacrylamide gel as described in Materials and Methods. Sub refers to the 5'-labeled starting substrate. 73b, PDC I, and PDC II refer to the products of the endonuclease reaction, i.e., the 73-base fragment and protein-DNA complexes I and II, respectively. Dimer refers to a dimer molecule of the starting substrate that is the result of a DNA ligase that eluted near the Rep68(I) peak. (B) Endonuclease, helicase, and DNA-binding assays of Rep68 ssDNA-agarose fractions. The Rep68 ssDNA-agarose fractions shown in panel A were assayed for the presence of terminal hairpin-binding activity ($\blacktriangle$), *trs* endonuclease activity ($\bullet$), and DNA helicase activity ($\blacklozenge$) as described in Materials and Methods. The products of the reactions were counted by using a scanning gas flow counter. The products of the endonuclease reaction plotted in panel B were heat denatured prior to electrophoresis to determine the total amount of nicked substrate produced. (C) The protein in 90 μ l of the indicated fractions was precipitated with acetone and immunoblotted as described in Materials and Methods. Lanes marked WCE contain a whole-cell extract of cells infected with adenovirus plus AAV.

FIG. 5. (A) ssDNA-agarose chromatography of the Rep78 DEAE fraction from a 1 M nuclear extract. Aliquots (3 μ l) of the indicated fractions were assayed under standard *trs* endonuclease reaction conditions. After incubation, the reaction products without heat denaturation were electrophoresed on a 6% nondenaturing polyacrylamide gel as described in Materials and Methods. Sub refers to the 5'-labeled starting substrate. 73b, PDC I, and PDC II refer to the products of the endonuclease reaction, i.e., the 73-base fragment and protein-DNA complexes I and II, respectively. Dimer refers to a dimer molecule of the starting substrate that is the result of a DNA ligase that eluted near the Rep78 peak. (B) Endonuclease, helicase, and DNA-binding assays of Rep78 ssDNA-agarose fractions. The Rep78 ssDNA-agarose fractions shown in panel A were assayed for the presence of terminal hairpin-binding activity (Δ), *trs* endonuclease activity ($\circ$), and DNA helicase activity ($\diamond$) as described in Materials and Methods. The products of the reactions were counted by using a scanning gas flow counter. The products of the endonuclease reaction plotted in panel B were heat denatured prior to electrophoresis to determine the total amount of nicked substrate produced. (C) The protein in 90 μ l of the indicated fractions was precipitated with acetone and immunoblotted as described in Materials and Methods. The lane marked WCE contains a whole-cell extract of cells infected with adenovirus plus AAV.

comigrated with a peak of DNA-binding activity that was specific for the AAV terminal hairpin fragment (Fig. 4B, triangles), a peak of *trs* endonuclease activity (Fig. 4B, circles), and a peak of DNA helicase activity (Fig. 4B, diamonds). The elution profiles of the helicase, endonuclease, and binding activities were not completely identical. The difference in elution profiles was apparently due to contaminating activities in the side fractions of the Rep protein peak, which inhibited the various activities of the Rep68 protein to different extents (see below).

A similar elution profile was seen during ssDNA-agarose chromatography of Rep78 (Fig. 5). A DNA helicase activity (Fig. 5B, diamonds), a DNA-binding activity specific for the AAV terminal hairpin (Fig. 5B, triangles), and the *trs* endonuclease activity (Fig. 5C, circles) all comigrated with the peak of the Rep78 protein (Fig. 5C, fractions 13 to 22). In contrast to Rep68(I), however, a second peak of terminal hairpin-binding activity was detected in the Rep78 eluants (Fig. 5B, fractions 20 to 31). This second peak of DNA-binding activity was also specifically inhibited by unlabeled AAV terminal hairpin DNA (data not shown) and was also associated with a peak of DNA helicase activity (Fig. 5B). However, the second peak did not comigrate with *trs* endonuclease activity or comigrate with the peak of the Rep78 protein. As yet, we have not identified the protein responsible for the second peak of DNA-binding and helicase activity. Presumably, the second peak consisted of one of more cellular enzymes which could interact with parvovirus DNA. A precedent for this kind of protein has recently been reported by Metcalf et al. (26).

Finally, we analyzed the products of the *trs* endonuclease reaction without heat denaturation prior to electrophoresis (Fig. 4A and 5A). We had previously noticed (15) that two kinds of protein-DNA complexes (PDCs) are formed as products during the *trs* endonuclease reaction with Rep68(I), a major slower-migrating form (PDC II) and a minor faster-migrating species (PDC I) (Fig. 4A and 5A). The most abundant protein-DNA complex, PDC II, consists of at least 1 mol of the Rep protein covalently attached via a tyrosine-phosphate linkage to a nicked hairpinned DNA molecule (Fig. 1C) (15, 32, 33). The two complexes are not interconvertible by heat denaturation (15), and a more complete analysis of the composition of these complexes will be presented elsewhere (15a). Here, we note that both Rep68 and Rep78 can form both kinds of complexes (PDC I and PDC II) and that the peak of complex formation comigrates with *trs* endonuclease activity and the peak of the Rep protein during ssDNA-agarose chromatography of Rep68 and Rep78 (Fig. 4A and 5A). Furthermore, the ability to form the less abundant PDC I form is found only in the fractions with the highest Rep68 or Rep78 concentration.

Several other activities were found within some of the ssDNA-agarose fractions that contained Rep78 or Rep68 protein. An unidentified exonuclease activity eluted just prior to the peak of Rep78 activity in fractions 7 to 10 (data not shown). We could not rule out the possibility that the exonuclease activity affected the elution profile of Rep78. In addition, a cellular ligase activity eluted near the peaks of both the Rep68 and Rep78 proteins. Evidence of the ligase activity can be seen in Rep78 fractions 16 to 19 (Fig. 5A) and Rep68 fractions 10 to 12 (Fig. 4A). In both cases a novel product can be seen in the *trs* endonuclease reactions, which we subsequently identified to be a ligated dimer of the starting substrate. The ligase activity also may have affected the elution profiles of either the *trs* endonuclease or helicase activities of both Rep proteins. We note that the ligase was

FIG. 6. Sedimentation analysis of Rep68(I) by sucrose gradient centrifugation. The pooled ssDNA-agarose fractions of Rep68(I) were sedimented through a 10 to 30% linear sucrose gradient as described in Materials and Methods. A total of 35 fractions were collected. Odd-numbered fractions were assayed for *trs* endonuclease activity ($\circ$), DNA helicase activity ($\diamond$), and terminal hairpin DNA-binding activity ($\triangle$). The products of the assay were quantified by gas flow counting. In the *trs* endonuclease assays the products were analyzed on a 6% polyacrylamide gel without heat treatment prior to gel loading. Both the amount of the PDC II product (Fig. 1C and 4A) and the amount of 73-base fragment were measured, but only the amount of 73-base product is plotted. The ability to form the protein-DNA complex comigrated with the ability to form the 73-base fragment (data not shown). A sedimentation marker (BSA; 68 kDa) was run in a parallel centrifuge tube, and its mobility is indicated by the arrow.

not a detectable contaminant in the Rep68 or Rep78 fractions obtained from the cytoplasmic and 0.2 M nuclear extracts (data not shown).

Sucrose gradient centrifugation of Rep68(I). To confirm that the Rep DNA helicase, *trs* endonuclease, and terminal hairpin-binding activities were all intrinsic properties of the same protein, we fractionated the pooled ssDNA-agarose fractions of Rep68(I) by sucrose gradient centrifugation (Fig. 6). The site-specific DNA-binding, *trs* endonuclease, and DNA helicase activities cosedimented at the monomer protein position of Rep68 on the sucrose gradient. We did not detect any DNA helicase or endonuclease activity in other fractions of the gradient, suggesting that the endonuclease and helicase activities were both intrinsic properties of the Rep68 protein and that Rep68 exists predominantly as a monomer protein in solution. Finally, the ratio of M13 DNA helicase activity to *trs* endonuclease activity was essentially the same for both Rep68(I) and Rep78 (4.6 and 4.9, respectively) when the peak fractions were pooled from their respective ssDNA agarose columns.

Recognition of the same *trs* site by Rep78 and Rep68. It was known that *in vivo*, the terminal resolution nicking sites were heterogeneous; the major site was located at AAV nucleotide 124 (50% of the nicked molecules), and two minor sites were located at nucleotide 125 (35%) and nucleotide 123 (15%) (10). Our previous results with purified Rep68(I) showed that only the site at nucleotide 124 was cut *in vitro* (15). To determine whether there was any difference in the nicking sites of Rep78 and Rep68, we measured the sizes of the nicked products produced by the DEAE and ssDNA Rep78 and Rep68 pooled fractions. The results (Fig. 7) showed that all of the Rep fractions produced a major nicking product that was 73 bases long and was the result of

FIG. 7. Comparison of the cleavage sites of Rep78 and Rep68. The 5'-labeled *Xba*I NE terminal fragment was incubated with Rep78 and Rep68 pooled DEAE-cellulose (DE) and ssDNA-agarose (SS) fractions obtained from the 1 M nuclear extract. After incubation in the standard *trs* endonuclease reaction, the products were treated with proteinase K as described in Materials and Methods and electrophoresed on a 6% polyacrylamide-8 M urea sequencing gel. The lane marked G+A contained a G+A sequencing ladder which was generated from the starting substrate by chemical modification (24). The lane marked NE contained a reaction in which Rep was omitted. Sub and 73b indicate the positions of the starting substrate and the 73-base product, respectively.

a nick at nucleotide 124. We could not detect any significant cutting at the flank sites. We also note that the less pure Rep78 DEAE fraction resulted in a minor band which ran at a higher-molecular-weight position. We did not investigate the origin of this band further. We concluded that the heterogeneity in the *trs* site that is seen *in vivo* was not likely to be the result of a difference in the nicking sites preferred by Rep78 and Rep68.

Binding of the four Rep proteins to the hairpinned AAV terminal repeat. We could not detect a stable binding complex when Rep52 or Rep40 was incubated with the AAV terminal hairpin fragment (Fig. 8). This was apparently not due to an inhibitor present in the Rep52 or Rep40 fractions, because less pure fractions which contained mixtures of Rep68 and Rep40 or Rep78 and Rep52 produced the same gel shift complexes that were seen with fractions that contained only Rep78 or Rep68. Both Rep78 and Rep68 were capable of binding specifically to the AAV terminal hairpin repeat (Fig. 8), confirming our earlier results (14). We concluded that the Rep52 and Rep40 proteins by themselves bind poorly, if at all, to the terminal AAV sequence in the hairpin configuration.

Nucleotide and divalent cation requirements for the *trs* endonuclease reaction. We showed previously that the presence of ATP was required for the *trs* endonuclease reaction (15, 33). In addition to ATP, several other nucleoside triphosphates were found to be efficient cofactors for Rep68(I). These included, in order of preference, CTP, GTP, and UTP (Fig. 9A). In contrast, only ATP was an efficient

FIG. 8. Site-specific DNA-binding activity of the AAV Rep proteins. The ability of the four AAV Rep proteins to bind to the AAV terminal hairpin sequence was measured by the gel shift assay as described in Materials and Methods. Pooled fractions obtained during the purification of the Rep proteins from the 1 M S-100 extract (Fig. 2B) were incubated with the 5'-labeled NE *Xba*I fragment. The fractions used are the same as those in Fig. 3C and are described in the text. They include the phenyl-Sepharose (PS), DEAE-cellulose-bound (DE Bound), and DEAE-cellulose flow-through (DE FT) fractions. The numbers in parentheses indicate the Rep proteins contained in each fraction (Fig. 3C). UNB is the unbound substrate.

cofactor for Rep78 (results not shown). As reported previously (15, 33), in the absence of any nucleoside triphosphate, no endonuclease activity could be detected with either Rep68 (Fig. 9A) or Rep78 (results not shown). Nonhydrolyzable nucleotide analogs (ATP- γ -S or AMP-PNP), as well as nucleoside mono- and diphosphates, also produced no detectable endonuclease activity. This suggested that hydrolysis of the nucleoside triphosphate cofactor was essential for site-specific nicking activity.

Only Mg^{2+} could efficiently support the *trs* endonuclease reaction (Fig. 9B). Mn^{2+} supported the reaction at approximately 1 to 5% the level that was seen with Mg^{2+} and no endonuclease activity was seen in the presence of Zn^{2+} or Ca^{2+} .

DISCUSSION

We have described a purification procedure by which the four AAV Rep proteins, Rep78, Rep68, Rep52, and Rep40, can be separated. The separation and partial purification of each Rep protein allowed us to compare some of the biochemical activities of the four proteins. In previous work (14, 15, 33, 34) we demonstrated that Rep68 contained at least four biochemical activities: (i) a DNA-binding activity specific for the AAV terminal hairpin sequence, (ii) an ATP-dependent *trs* endonuclease activity, (iii) the ability to bind covalently to the 5' end of the nick at the *trs* site, and (iv) a DNA helicase activity. In this report we have demonstrated that Rep78 has at least three of these activities, i.e., the terminal hairpin-binding, DNA helicase, and *trs* endonuclease activities. Our preliminary evidence (not shown) indicates that Rep78 is also capable of covalently attaching to the 5' end of the nick. Thus, either of the two p5 Rep proteins could play a role in the terminal resolution step

FIG. 9. (A) Nucleotide cofactor requirements for the *trs* endonuclease reaction. The ability of the indicated nucleotide cofactors to support cleavage of the 5'-labeled terminal *Xba*I fragment was tested under standard *trs* endonuclease reaction conditions by using Rep68(I) obtained from a 0.2 M nuclear extract. The final concentration of all nucleotides was 0.4 mM. All other conditions were as described in the legend to Fig. 7. The starting substrate and 73-base cleavage product (Sub 73, respectively) are indicated to the left of the figure. Lane 10 contains a reaction in which no nucleotide cofactor was added. Lane 1 contains a C+T sequencing ladder generated from the starting substrate by chemical modification. (B) Divalent cation requirement for cleavage at the *trs* site. The 5'-labeled terminal *Xba*I fragment was incubated with Rep68(I) isolated from a 1 M nuclear extract in a reaction mixture containing 5 mM $MgCl_2$, $MnCl_2$, $ZnCl_2$, $CaCl_2$, or no divalent cation (lane -). After incubation at 37°C for 1 h, the reaction mixtures were heated for 5 min at 100°C and electrophoresed on a nondenaturing 6% polyacrylamide gel.

during AAV DNA replication. We have observed only three differences thus far between the purified Rep78 and Rep68 fractions *in vitro*. First, the chromatographic behavior of the two proteins on phenyl-Sepharose columns was different, presumably because the amino acids encoded by the intron (amino acids 578 to 621) contain a hydrophobic domain that is present on the surface of the Rep78 protein. Second, Rep68 could use several nucleoside triphosphates in addition to ATP as cofactors in the *trs* endonuclease reaction, whereas Rep78 appeared to be capable of using only ATP. Finally, purified Rep68 produced a qualitatively different gel shift pattern than Rep78 did. Whereas the Rep68 gel shift pattern consists of three to four discrete bands, the Rep78 pattern was a higher-molecular-weight heterogeneous smear (Fig. 7). The significance of the last two observations was not clear.

In contrast to the two p5 Rep proteins (Rep68 and Rep78), we could not detect any binding activity in the p19 Rep protein (Rep52 or Rep40) fractions which was specific for the AAV terminal hairpin. So far, we also have not been able to

detect *trs* endonuclease or DNA helicase activity in the p19 Rep protein fractions (not shown). Elsewhere, we showed that a large excess of Rep52 and Rep40 that was present in crude cytoplasmic extracts did not significantly affect the terminal resolution reaction and that neither Rep52 nor Rep40 was covalently associated with the *trs* site (33). Genetic studies also suggested that the p19 Rep proteins are not essential for the synthesis of duplex AAV replicative forms (9). Taken together, these observations suggest that the p19 Rep proteins are not required for AAV DNA replication. Finally, the lack of DNA-binding activity in the Rep52 and Rep40 fractions also suggests that at least a part of the DNA-binding domain of the p5 Rep proteins must be in the N-terminal amino acid sequence between the p5 and p19 translation initiation sites (amino acids 1 to 214).

The sedimentation of Rep68 in a sucrose gradient suggested that the protein existed predominantly as a monomer in solution. However, it is important to point out that this result does not exclude the possibility that Rep68 forms a homodimer (or a more complex multimer) under different conditions. It is also possible that the Rep protein assembles as a dimer or a multimer on the substrate before nicking occurs. Several lines of indirect evidence suggest that the p5 Rep proteins may be capable of forming either homo- or heteromultimeric protein complexes. First, two forms of Rep68 could be distinguished by their ability to bind to ssDNA-agarose. Rep68(II), which did not bind to ssDNA, was found to contain the same hairpin-binding, *trs* endonuclease, and DNA helicase activities that were associated with Rep68(I) (data not shown). However, when Rep68(II) was analyzed by sucrose gradient centrifugation, all three activities were found not only at the monomer Rep68 position but also at positions of higher molecular weight (data not shown). We do not know yet whether Rep68(II) aggregates with itself or forms complexes with cellular proteins. Second, higher-molecular-weight complexes that contain one or more Rep proteins could be seen by immunoblotting in whole-cell extracts (Fig. 3) and in fractions that contain high concentrations of Rep78 (Fig. 5C). Finally, it may not be fortuitous that both Rep78 and Rep68 copurified with a cellular ligase activity (Fig. 4A and 5A). The apparent molecular mass of the ligase by sucrose gradient centrifugation, 68 kDa, corresponds to the molecular mass of a previously described cellular DNA ligase (38). Although we have no reason to believe that ligase activity is essential for AAV DNA replication, it may play a role in the sealing of inappropriate nicks made during AAV DNA replication or it may be involved in the integration or excision of AAV DNA from cellular chromosomes. It will be necessary to develop a complete in vitro AAV DNA replication system to determine the precise role of cellular proteins and Rep complexes in AAV DNA replication.

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