A Biochemical Characterization of the Adeno-associated Virus Rep40 Helicase*

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Roy F. Collaco, Vivian Kalman-Maltese, Andrew D. Smith, John David Dignam, and James P. Trempe‡

From the Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, Ohio 43614

The human adeno-associated virus (AAV) has generated much enthusiasm as a transfer vector for human gene therapy. Although clinical gene therapy trials have been initiated using AAV vectors, much remains to be learned regarding the basic mechanisms of virus replication, gene expression, and virion assembly. AAV encodes four nonstructural, or replication (Rep), proteins. The Rep78 and Rep68 proteins regulate viral DNA replication, chromosomal integration, and gene expression. The Rep52 and Rep40 proteins mediate virus assembly. To better understand Rep protein function, we have expressed the Rep40 protein in *Escherichia coli* and purified it to near homogeneity. Like the other Rep proteins, Rep40 possesses helicase and ATPase activity. ATP is the best substrate, and Mg

The AAV Rep proteins are pleiotropic effectors of viral replication and gene expression. Rep78/68 are required for viral DNA replication, integration of the provirus into chromosome 19, and regulation of viral gene expression. The N-terminal 224 amino acids of Rep78/68 are involved in interaction with Rep-binding sites (RBS) in the viral origin of DNA replication and in the chromosome 19 integration site. Upon interaction with either RBS, the protein makes a site-specific, strand-specific, single-strand cut at the terminal resolution site (trs) ~10 base pairs away from the RBS. This nicking is required for viral DNA amplification and integration into the chromosome 19 locus. Rep78/68 repress AAV mRNA transcription from the p5 promoter (5). These proteins also trans-activate mRNA transcription from the p19 and p40 promoters (6, 7). The Rep52/40 proteins play roles in regulation of gene expression and repress p5 transcription in the absence of Ad infection. Rep52/40 are not required for viral DNA replication but play roles in assembly of the viral DNA into a preformed virion capsid (8, 9). The Rep proteins share a common purine nucleotide-binding site. Rep78/68 and Rep52 function as helicases and ATPases in *in vitro* assays (10–13). All four Rep proteins share conserved amino acid motifs common to helicase superfamily 3 (14). The helicase activity of Rep78/68 may be involved in unwinding the covalently closed end of the linear viral DNA after making the site-specific nick at the trs site. The helicase activity of the smaller Rep proteins is involved in the insertion of single-stranded viral DNA into a preformed virion (9). Rep78/68 and Rep52 have 3′–5′ helicase polarity (11, 13), and Rep68 func-

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‡ To whom reprint requests should be addressed: Dept. of Biochemistry and Molecular Biology, Medical College of Ohio, Block Health Science Bldg., Rm. 408, 3303 Arlington Ave., Toledo, OH 43614. Tel.: 419-383-4103; Fax: 419-383-6228; E-mail: jtempe@mco.edu.

1. The abbreviations used are: AAV, adeno-associated virus; RBS, Rep-binding site(s); AMP-PNP, 5′-adenylylimidophosphate; AMP-PCP, β,γ-methyleneadenosine-5′-triphosphate; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholino propane sulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine; CAPS, 3-cyclohexylamino propane sulfonic acid; MBP, maltose-binding protein.

The absence of a helper virus in differentiating keratinocytes (3). Unlike the other human and animal parvoviruses, AAV is nonpathogenic. In a nonpermissive environment, AAV integrates its genome into the long arm of chromosome 19 (1). The natural defectiveness, nonpathogenicity, and ability of AAV to integrate into what is an apparently innocuous location in chromosome 19 are appealing characteristics of the virus as a human gene therapy vector.

AAV encodes two genes: a capsid, or structural, gene and a nonstructural, or replication, gene. The replication gene encodes four replication (Rep) proteins from the same translation open reading frame. Rep78, Rep68, Rep52, and Rep40 are named for their apparent molecular weights as estimated from their mobility in SDS-PAGE (4). Rep78/68 are translated from mRNAs that originate from a transcription promoter at map unit 5 (P5), whereas the Rep52/40 proteins are translated from mRNAs that originate from a transcription promoter at map unit 19 (P19). The Rep68 and Rep40 proteins differ from their longer counterparts in that they are translated from spliced mRNAs from the P5 and P19 promoters, respectively. Splicing removes 92 amino acid residues from the carboxyl termini of the Rep78 and Rep52 proteins and replaces them with 9 amino acids located at the C termini of Rep68 and Rep40.

The AAV Rep proteins are pleiotropic effectors of viral replication and gene expression. Rep78/68 are required for viral DNA replication, integration of the provirus into chromosome 19, and regulation of viral gene expression. The N-terminal 224 amino acids of Rep78/68 are involved in interaction with Rep-binding sites (RBS) in the viral origin of DNA replication and in the chromosome 19 integration site. Upon interaction with either RBS, the protein makes a site-specific, strand-specific, single-strand cut at the terminal resolution site (trs) ~10 base pairs away from the RBS. This nicking is required for viral DNA amplification and integration into the chromosome 19 locus. Rep78/68 repress AAV mRNA transcription from the p5 promoter (5). These proteins also trans-activate mRNA transcription from the p19 and p40 promoters (6, 7). The Rep52/40 proteins play roles in regulation of gene expression and repress p5 transcription in the absence of Ad infection. Rep52/40 are not required for viral DNA replication but play roles in assembly of the viral DNA into a preformed virion capsid (8, 9). The Rep proteins share a common purine nucleotide-binding site. Rep78/68 and Rep52 function as helicases and ATPases in *in vitro* assays (10–13). All four Rep proteins share conserved amino acid motifs common to helicase superfamily 3 (14). The helicase activity of Rep78/68 may be involved in unwinding the covalently closed end of the linear viral DNA after making the site-specific nick at the trs site. The helicase activity of the smaller Rep proteins is involved in the insertion of single-stranded viral DNA into a preformed virion (9). Rep78/68 and Rep52 have 3′–5′ helicase polarity (11, 13), and Rep68 func-
tions as a RNA/DNA helicase (12). Rep proteins with mutations in the purine nucleotide-binding pocket are inactive in helicase assays and are inactive in DNA replication, virus assembly, and regulation of gene expression (8).

Preliminary enzymatic characterizations of the Rep78/68 and Rep52 helicase activities have been performed (10–13). However, no such studies have been published for the Rep40 protein. To present an analysis of the enzymatic activities of the Rep40 protein, Rep40 is similar to Rep52 with respect to its nucleotide and divalent cation requirements but differs in that it unwinds double-stranded DNA substrates with 3′ or 5′ single strand tails as well as substrates with blunt ends. A mutant of Rep40 with a Lys to His substitution in the carboxyl terminus. It was purified using the same procedure as for Rep40 and was judged enzymatically active by its ability to unwind a helicase substrate with a 5′ single-stranded end.

Helicase Substrates—To prepare the standard M13 substrate, a 25-µl reaction containing 0.2 pmol of M13 single-stranded DNA and 0.3 pmol of Primer DN (5′-GTTTTTCCAGTCAGCAC-3′) in 67 mM potassium phosphate (pH 7.5), 6.7 mM MgCl2, and 1 mM dithiothreitol was heated to 95 °C for 10 min and cooled gradually to room temperature. TTF and dATP were added to 0.75 mM, 50 µCi of [32P]dATP (Amersham Biosciences) and 5 units of Klenow DNA polymerase (Promega) were added to a final volume of 50 µl. The reaction was incubated at 37 °C for 75 min. Unlabeled dATP was added to 0.75 mM, and the reaction was incubated at 37 °C for 15 min. This reaction extends the 17 nut-DN primer to a length of 26 nt. The reaction mixture was applied to a Sephadex G-25 (Superfine) spin column and centrifuged at 1000 rpm for 10 min. Unincorporated nucleotides were removed. The purified, labeled DNA was brought to 200 µl (7.5 mM Tris-HCl, pH 8, 0.75 mM EDTA, and 50 mM NaCl). For preparation of the M13 directional substrate, a 25-µl reaction containing 0.2 pmol of M13 single-stranded DNA and 0.3 pmol of Primer C (5′-GGAGATCCCGGCTACCGGCTGATATCACTAAGTCGATCCGGCCTATGACTGCTTGTTATACCCGCTCACACT-CCACACAAC-3′) in 67 mM potassium phosphate (pH 7.5), 6.7 mM MgCl2, and 1 mM dithiothreitol was heated to 95 °C for 10 min and cooled gradually to room temperature. The primer-M13 DNA was digested with EcoRI, TFP (0.75 mM), 110 µCi of [γ-32P]dATP, and 5 units of Klenow DNA polymerase I were added in a volume of 50 µl. The reaction was incubated at 37 °C for 75 min. Unlabeled dATP was added to 0.75 mM, and the reaction was incubated for 15 min. Unincorporated nucleotides were removed with a Sephadex G-25 spin column as described above. The purified, labeled DNA was brought to 200 µl (7.5 mM Tris (pH 8), 0.75 mM EDTA, and 50 mM NaCl). Unwinding of this substrate in the 3′→5′ and 5′→3′ directions yields a radioabeled 28-mer and 70-mer, respectively. For preparation of the blunt-end substrate, Primer C was end-labeled with [γ-32P]ATP and T4 polynucleotide kinase at 37 °C for 90 min. The labeled primer was purified on a Sephadex G-25 spin column as described earlier and annealed to an equimolar amount of Primer G (the complement of Primer C) by incubating at 75 °C for 10 min and cooled gradually to room temperature. The annealed product (Substrate X9) was applied to a 15% acrylamide gel with 90 mM Tris borate, 2 mM EDTA (pH 8.0) and electrophoresed at 100 V for 45–60 min. The gel was exposed to film for 20 min, the predominant labeled band was excised, and the DNA was eluted using a Bio-Rad electrophoretic (model 422). For electrophoresion, the buffer used was 0.25× the buffer used in the electrophoresis (22.5 mM Tris borate, 0.5 mM EDTA). After elution, the sample was concentrated and further purified on a Sephadex G-25 spin column to remove excess EDTA. The purified sample was digested with EcoRI, yielding a blunt end, labeled 28-mer with a 5′-overhang at the other end. This overhang is filled in by incubation with 0.75 mM dATP, 0.75 mM TTP, and 5 units of Klenow DNA polymerase (Promega) at 75 °C for 75 min. The blunt-end substrate (Substrate X5) was gel-purified, electroeluted, and column-purified as described earlier. Unwinding this blunt-ended substrate yields a 28-nucleotide product.

Helicase Assay—The standard 20-µl helicase assay contained 2 fmol of substrate, 25 mM MES (pH 6.5), 2 mM ATP, 2 mM MgCl2, 1 mM dithiothreitol, 10 µl albumin (1 mg/ml), and 0.1 µg MBPRep52 expressed as a maltose-binding protein fusion protein unless otherwise noted in the figure legends. AMP-PNP and AMP-PCP were used at a concentration of 2 mM. For the blunt-ended substrate, the helicase reaction was supplemented with ATP and MgCl2 at 2 mM each. The reactions were incubated at 30 °C for 35 min, and 2 µl of sample loading solution (0.4% orange G, 0.03% bromphenol blue, and 20% glycerol) was added to each tube. The reactions were run on a 10–20% PAGE gel for 1.5 h at 100 V. The gels were stained with 0.5% Coomassie Brilliant Blue R-250, destained in 10× destaining buffer (7.5% acetic acid, 40% methanol), and scanned.
acrylamide gel with 90 mM Tris borate, 2 mM EDTA (pH 8.0) and electrophoresed at 100 V for 45–60 min. Gels were placed in 40% methanol, 10% acetic acid for 10 min, dried, and placed on x-ray film for 15–20 h. The dependence of helicase activity on pH was examined in sodium acetate (pH 4.7), MES (pH 6 and 6.5), MOPS (pH 7), Tris-HCl (pH 7.5 and 8), Bicine (pH 8.5 and 9), and CAPS (pH 10). Radioactivity in dried gels was determined using a Typhoon phosphor imager system. The percentage of unwound product was determined by dividing the counts in the product band by the total counts of the product in the lane containing the boiled substrate.

**ATPase Assay**—ATPase activity was determined in a 30-μl reaction containing 25 mM Tris-HCl (pH 7.5), 25 mM NaCl, 2 mM MgCl₂, 10 μg/ml bovine serum albumin, 1% glycerol, 2 mM Mg-ATP with [γ-³²P]ATP at 1.5–2.8 cpm pmol⁻¹. [γ-³²P]ATP was purified by chromatography on Q-Sepharose. For ATP substrate saturation experiments, Mg-ATP was varied from 0.1 to 10 mM. For assays performed in the absence of DNA, 1.6 μg of Rep40 was used, and the reactions were incubated at 30 °C for 16 min. For assays performed in the presence of DNA (20 μg), 0.8 μg of Rep40 was used, and the reactions were incubated at 30 °C for 3 min. Reactions were terminated by the addition of 60 μl of 25 mM sodium acetate (pH 4.5), 20 mM EDTA followed by the addition of 90 μl of a Norit A suspension (12.5% (w/v) in H₂O). Samples were mixed, incubated at room temperature for 15 min, and centrifuged twice to remove Norit A. Radioactivity in the supernatant was determined by liquid scintillation. Data from ATP substrate saturation experiments were fit by nonlinear least squares with Microcal Origin using the relationship

\[ v = \frac{V}{1 + \frac{K_{ATP}}{[ATP]}} \]

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for these parameters are reported ± S.D. of the fit.

**RESULTS**

**Expression and Purification of Wild Type Rep40 and Rep40PNB**—Vectors that express the wild type Rep40 protein and a mutant protein, Rep40PNB, were constructed as described above. Rep40PNB has a single amino acid change of a Lys to His at residue 340. This substitution in the Rep78/68 and Rep52 proteins inactivates Rep protein helicase activity and prevents Rep78/68 from replicating AAV DNA (11). Both proteins contain a His₉ tag at the carboxyl terminus. The proteins were expressed and purified from 4 liters of midlog phase culture. Cell lysates were fractionated over a Ni²⁺-nitrilotriacetic acid resin. Because the eluted protein from the Ni²⁺ column was contaminated with numerous bacterial proteins and nonspecific phosphatases, two additional chromatography steps, MonoQ and MonoS, were performed. Fig. 1 shows the results of SDSPAGE on the final purified fractions of Rep40 (lane 2) and Rep40PNB (lane 4). Both proteins are estimated to be greater than 90% homogeneous. Typically, a 4-liter culture yields ~1.5 mg of protein after the three-column fractionation procedure.

**Cofactor and Enzyme Reaction Requirements for Helicase Activity**—DNA helicase activity was initially studied using a M13 single-stranded DNA template annealed to a radioactively labeled, 26-base oligonucleotide. Helicase assays using this substrate revealed that the activity was dependent upon both ATP and Mg²⁺ (Fig. 2A, lane 5). Nonhydrolyzable analogs AMP-PCP and AMP-PNP did not support enzyme activity (Fig. 2A, lanes 7 and 8). Adding ATP to the nonhydrolyzable analogs at a concentration of 2 mM each restored enzymatic activity (Fig. 2A, lanes 9 and 10). The inability of the analogs to compete with ATP suggests that they may bind to the purified helicase that has a lower affinity than ATP. Purification of Rep40 revealed one prominent protein species (Fig. 1). However, to rule out the possibility that a co-purifying contaminant was responsible for the helicase activity, we performed helicase assays using purified Rep40PNB. As anticipated, the mutant Rep40PNB protein did not possess enzymatic activity (Fig. 2A, lane 11).

The majority of helicases function as multimeric enzymes. To
determine whether Rep40 functions as a multimer, we tested whether the Rep40PNB mutant protein could function as a dominant negative inhibitor of helicase activity. Keeping the enzyme concentration constant, the ratio of wild type to mutant protein was altered. When the wild type protein was in a 3:1 excess over the mutant protein, helicase activity was evident (Fig. 2B, lane 4). When there was a 1:1 ratio of wild type to mutant protein or more mutant than wild type protein, there was a nearly complete inhibition of enzyme activity (Fig. 2B, lanes 5–7). Maximal enzyme activity is attained at 16 ng of Rep40 in the standard helicase assay, and low activity is observed down to 1 ng of protein (Fig. 2C). Therefore, helicase activity should have been detected at the 20- and 10-ng levels used in the mixing experiment (Fig. 2B). This result can be interpreted in one of two ways: either the enzyme functions as a multimer because the mutant form of the protein acted as a dominant negative component of the complex to inactivate enzyme activity, or the inactive protein bound to the DNA, preventing the active protein from gaining access to the substrate.

To test enzyme stability, the enzyme was heated for 30 min at 30, 37, and 42 °C for 30 min prior to the enzyme reaction. After the 30-min preincubation, the helicase substrate was added, and the reaction was allowed to continue. This experiment demonstrated that the enzyme is stable at 30 and 37 °C but is inactivated at 42 °C (Fig. 2B, lanes 8–10). We have also observed no detectable loss of helicase activity after more than five freeze-thaw cycles (data not shown).

We also examined various nucleotide substrate and divalent metal ions in helicase assays. The nucleotides were used at 0.5 mM, and ATP was the most efficient nucleotide for activity followed by CTP, GTP, and dATP, which were approximately half as efficient as ATP (Fig. 3B). UTP, dCTP, dGTP, and TTP were only minimally active in the assay. The enzyme worked with either Mg2+ or Mn2+ (Fig. 3A). The Rep52 enzyme has similar preferences in nucleotide and metal cofactors (11).

Ranges of Mg-ATP, NaCl, and temperature conditions were examined to determine optimal conditions for helicase activity on the M13 26-nt substrate. The optimal concentration for Mg-ATP was 1 mM (Fig. 4A). These assays were not performed under initial rate conditions, thus precluding an assessment of $K_m$ for ATP in the helicase reaction. The temperature optimum was 25 °C (Fig. 4B). The enzyme was sensitive to NaCl concentrations, with the optimal activity occurring at a concentration range of 0–25 mM (Fig. 4C).

Rep Helicase Substrate Analysis—To determine the directionality of helicase action, we used M13 DNA that was annealed to a 90-base oligonucleotide. The duplex was digested with EcoRI and the DNA products were radiolabeled by fill-in reactions with Escherichia coli Klenow DNA polymerase and radioactive nucleotides. This procedure produces a full-length linear M13 DNA annealed to 28-base and 72-base oligonucleotides at each end of the M13 DNA. All three DNA species are radiolabeled. If the 28-mer is removed, then the helicase would have to function in a 3’–5’ direction. If the 72-mer is removed, then the helicase would function in a 5’–3’ direction. Incubation with increasing amounts of the wild type or PNB mutant version of the Rep40 protein demonstrated that the 28- and 70-base oligonucleotides were both removed by the wild type form of the protein (Fig. 5A). Lower amounts of Rep40 also did not reveal a preference for either substrate (data not shown). No activity was observed over the enzyme-free reaction for the Rep40PNB protein. This result is in contrast to that observed with other AAV Rep proteins. Rep78/68 and a maltose-binding protein-Rep52 fusion protein, MBPRep52, demonstrate 3’–5’ directionality of helicase action on similar M13 type substrates (11, 13). To verify these results, we purified Rep68 (15) and MBPRep52 (11) using established methods and used these proteins with our directional substrate. MBPRep52 and Rep68 demonstrated 3’–5’ directionality, removing the 28-base oligonucleotide without releasing the 72-nucleotide product (Fig. 5B). Longer exposures of the film or increasing amounts of MBPRep52 or Rep68 did not reveal any unwinding of the 72-mer (data not shown). We have also expressed the Rep52 protein in E. coli without the MBP moiety and found that it also displays only 3’–5’ helicase activity (data not shown). Given the directionality of the helicase activities of the Rep68 and MBPRep52 proteins and the amino acid identity shared between all of the AAV Rep proteins, we believe that the directionality of Rep40 is also in the 3’–5’ direction. A 5’–3’ direction would be the apparent result if Rep40 binds to the blunt end of the 72-nt M13 substrate and unwinds the duplex. Thus, the apparent 5’–3’ directionality observed in Fig. 5B, lane 4, may be due to Rep40 acting on the blunt end of the 72-mer. To verify that Rep40 can unwind a blunt end DNA substrate, a 28-bp substrate was prepared. Rep40 unwind the blunt end substrate, whereas Rep52 did not unwind the substrate (Fig. 5C). These results indicate that the Rep40 helicase is less stringent in its requirements for DNA unwinding and will unwind DNA with a 3’-overhanging end or blunt, fully duplex ends.

Characterization of Rep40 ATPase Activity—Helicase function is dependent upon ATP hydrolysis. We investigated ATPase activity in the presence of a variety of nucleic acids to determine whether DNA or RNA stimulated the enzyme. The addition of double- or single-stranded DNA stimulated ATPase activity, with single-stranded DNA showing slightly higher stimulation (results not shown). Several different types and lengths of single-stranded nucleic acid were analyzed to determine whether Rep40 has any sequence or size preferences for optimal ATPase activity. ATPase activity was stimulated slightly more that 2-fold with polyadenylic acid, 3-fold with...
total yeast RNA, and -4-fold with polyuridine (Fig. 6). Using single-stranded DNA of increasing lengths, there was a trend toward greater stimulation with increasing lengths of DNA. We have routinely observed a 7–8-fold stimulation of ATPase activity when using denatured, sonicated calf thymus DNA (Fig. 6). This DNA has a broad range of lengths between 500 and 800 bp. These results are in contrast to those observed with the Rep68 protein for which M13 DNA did not stimulate ATPase activity (13). Nucleic acid stimulation of Rep52 ATPase activity has not been examined. The pH maximum for both helicase and ATPase activity revealed maximal activity at pH 6.5 (data not shown).

ATPase activity in the presence and absence of single-stranded calf thymus DNA was determined over a range of ATP concentrations, under initial rate conditions (Fig. 7). The $K_{\text{ATP}}$ for the ATPase activity in the presence of DNA was $1.2 \pm 0.2$ and was comparable with that observed in the absence of DNA, $1.1 \pm 0.2$ (Table I). The $V_{\text{max}}$ was $220 \pm 10$ nmol/min/mg in the absence of DNA. In the presence of DNA, the rate increased 7–8-fold to $1,500 + 90$ nmol/min/mg. The value for $V_{\text{max}}$ derived from the data in Fig. 7 is consistent with the result with single-stranded calf thymus DNA shown in Fig. 6. The results shown in Fig. 6 suggest that Rep40 utilizes one class of binding sites for ATP for hydrolysis.

**DISCUSSION**

The extensive amino acid identity between the four AAV Rep proteins indicates that they all may perform similarly in enzymatic assays. Rep68 and a maltose-binding protein-Rep52 (MBPRep52) fusion protein have been most thoroughly analyzed of the four Rep proteins. The Rep68 protein contains 224 residues at its amino-terminal end that are absent from Rep40. This domain is responsible for the protein's site-specific DNA binding to the AAV inverted terminal repeat and chromosome 19 S1 sites (16, 19). The MBPRep52 protein contains the E. coli maltose-binding proteins fused to the amino terminus of the normal Rep52 and contains the unspliced carboxyl terminus of the Rep52 protein. Both of these elements are missing from Rep40. Our analyses of the enzymatic properties of the Rep40 protein indicate that this diminutive member of the AAV Rep protein family has distinct differences in its functional characteristics. The helicase activity of Rep40 was most active when using ATP as a nucleotide substrate. CTP, GTP, and dATP could function in place of ATP with -50% efficiency. This result is comparable with that observed with MBPRep52, where ATP was the most efficient nucleotide substrate for helicase activity, followed by CTP, GTP, and dATP (11). These same nucleotides also are utilized by the Rep68 helicase (13). Mg$^{2+}$ and Mn$^{2+}$ functioned as metal cofactors for Rep40 and also for MBPRep52 and Rep68. Thus for metal and nucleotide requirements, Rep40 and the MBPRep52 fusion proteins behave similarly.

Rep40PNB, which has a Lys to His change at amino acid 340 located in the Walker A box, is inactive in helicase and ATPase assays. A similar mutation in the MBPRep52 protein also results in an inactive protein (11). A striking difference between the two proteins is that the Rep40PNB protein inhibits helicase activity of the wild type protein, whereas the MBPRep52PNB does not alter the helicase activity of the wild type form of the fusion protein. The ability of Rep40PNB to inhibit Rep40 helicase suggests that the protein may normally function as a multimer requiring at least two units or that the mutant form of the protein, acting as a monomer, binds to DNA, preventing binding of the normal protein. Unlike Rep78/68, which forms an oligomer on the RBS in the AAV TR element (20), and Rep68, which has been reported to function as a dimer in helicase assays (13), Rep52/40 has been proposed to function as a monomer when unwinding DNA (11). Most hypothesized mechanisms of helicase action suggest that there are at least two DNA binding sites on the enzyme complex (21). Multiple DNA binding sites may be found on one protein molecule, but DNA helicases for which protein oligomerization has been examined appear to form dimers or hexamers (21). Thus, Rep40 may form at least a dimer during helicase action. However, an alternative explanation may be that the mutant protein binds to DNA more tightly and precludes binding of the wild type. The only way to determine whether the mutant binds more tightly is to determine its binding constant. The structural differences between the MBPRep52 and the carboxyl-terminal His-tagged Rep40 used here may be sufficient to explain the apparent functional differences. Rep52 possesses 92 amino acids at its carboxyl terminus that are absent from Rep40 due to mRNA splicing. The Rep52 C terminus contains zinc finger elements that bind Zn$^{2+}$ (22). The Rep52 C terminus may alter the protein conformation or DNA binding characteristics, resulting in a protein that has two DNA interaction domains, whereas Rep 40, which lacks the Rep52 C terminus, might require dimerization to function as a helicase.

Rep68, MBPRep52, and Rep40 all unwind DNA substrates that have 3’ single-strand ends, indicating a 3’–5’ polarity of unwinding. Surprisingly, Rep40 also unwinds a helicase substrate with a 5’ single-stranded end or a blunt end. Given the primary sequence identity between the proteins, it is likely that all four AAV Rep proteins unwind DNA in a 3’–5’ direction. The apparent lack of specificity for helicase substrate for Rep40 suggests that the enzyme acts on double-stranded DNA ends. In this respect, Rep40 is similar to the E. coli RecQ helicase. The RecQ helicase is important during homologous recombination and, in conjunction with RecA and SSB proteins, can initiate recombination events in vitro (23–25). RecQ is able to unwind a variety of DNA substrates, including joint molecules, single-stranded ends, and blunt ends (23–25). The ability
of Rep40 to act on fully double-stranded DNA ends may play an important role in the virus assembly process. Rep52/40 associate with preformed virion particles and mediate translocation of single-stranded viral DNA into the capsid structure. The 3'-end of the DNA is inserted first (9). In a fully double-stranded replicative form monomer AAV DNA molecule, Rep40 may bind to the end of the DNA and initiate unwinding. This would yield a flayed end with both 5' and 3' single-stranded regions. The single-stranded regions may then be acted upon by Rep52, and perhaps Rep40, resulting in translocation of the DNA into the capsid.

Our studies reveal significant differences between the Rep52 and Rep40 helicase. However, many other questions remain to be answered regarding the role of these proteins in virus replication and assembly. For example, why is there a requirement for Rep52 and/or Rep40 when the larger Rep78 and Rep68 proteins contain all of the primary sequence of the smaller proteins? There is also evidence that virus assembly occurs in the complete absence of Rep52/40 expression (9, 26). Is there a gain of function with the removal of the N terminus from Rep78/68 or C terminus from Rep78/52? Alternatively, removal of the RBS DNA binding domain from the N terminus of Rep52/40.
Rep78/68 may result in a loss of function, thus freeing Rep52/40 to act on other regions of AAV DNA. The various Rep proteins may also have differences in affinity for hairpin or single- or double-stranded DNA that dictate where they might exert their effects. Clearly, more rigorous biochemical analyses of purified Rep proteins is required to elucidate their functional differences and define their multiple roles in the AAV replication cycle.

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