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A Maltose-Binding Protein/Adeno-Associated Virus Rep68 Fusion Protein Has DNA-RNA Helicase and ATPase Activities

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The adeno-associated virus type 2 (AAV) Rep68 protein produced in *Escherichia coli* as a fusion protein with maltose-binding protein (MBP-Rep68Δ) has previously been shown to possess DNA-DNA helicase activity, as does the purified wild-type Rep68. In the present study, we demonstrate that MBP-Rep68Δ also catalyzes the unwinding of a DNA-RNA hybrid. MBP-Rep68Δ-mediated DNA-RNA helicase activity required ATP hydrolysis and the presence of Mg^{2+} ions and was inhibited by high ionic strength. The efficiency of the DNA-RNA helicase activity of MBP-Rep68Δ was comparable to its DNA-DNA helicase activity. However, MBP-Rep68Δ lacked the ability to unwind a blunt-ended DNA-RNA substrate and RNA-RNA duplexes. We have also demonstrated that MBP-Rep68Δ has ATPase activity which is enhanced by the presence of single-stranded DNA but not by RNA. The MBP-Rep68ΔNTP mutant protein, which has a lysine-to-histidine substitution at amino acid 340 in the putative nucleoside triphosphate-binding site of Rep68, not only lacks DNA-RNA helicase and ATPase activities but also inhibits the helicase activity of MBP-Rep68Δ. DNA-RNA helicase activity of Rep proteins might play a pivotal role in the regulation of AAV gene expression by AAV Rep proteins.

Adeno-associated virus type 2 (AAV) is a nonpathogenic human parvovirus which requires the coinfection of adenovirus or herpesvirus as a helper to enable its efficient replication (6). AAV has a linear single-stranded DNA genome which has 145-nucleotide-long inverted terminal repeats (42). The AAV genome contains two genes called *rep* and *cap* (14). The *rep* gene codes for four overlapping in-frame polypeptides referred to as Rep78, Rep68, Rep52, and Rep40 (30, 42). The *cap* gene encodes proteins called VP-1, VP-2, and VP-3 that form the capsid (7). The genome of AAV has three promoters called p_5, p_{19}, and p_{40}. The Rep78 and Rep68 proteins expressed from p_5 are required for viral DNA replication, while Rep52 and Rep40 expressed from p_{19} play a role in the accumulation of single-stranded progeny genomes used for packaging (8, 31). The VP-1, VP-2, and VP-3 capsid proteins are expressed from the p_{40} promoter (7).

Rep78 and Rep68 proteins have the ability to bind to the AAV inverted terminal repeats (2, 16) by recognition of a GCTC repeating motif (28, 46), cause a site-specific and strand-specific nick at the terminal resolution site (*trs*) (17, 41), covalently attach to the *trs* (40), and have a DNA-DNA helicase activity which may be involved in the unwinding of the inverted terminal repeats during replication of the AAV genome (17, 18). Rep78 and Rep68 have also been shown to negatively regulate the steady-state levels of mRNA transcribed from the p_5 and p_{19} promoters (3, 23, 25). Rep proteins also inhibit transcription from heterologous promoters (13, 24), inhibit cellular transformation by bovine papillomavirus (12) and adenovirus E1a plus an activated *ras* oncogene (20), and inhibit the replication of human immunodeficiency virus type 1 (HIV-1) (1, 29, 32, 36). Though there are several reports documenting the multifunctional abilities of AAV Rep proteins, the mechanisms by which they carry out their pleiotropic gene regulation effects are unknown.

In this report, we demonstrate two additional functions of Rep proteins, namely, the ability to unwind DNA-RNA hybrids and to hydrolyze ATP. We have used Rep68 protein produced in *Escherichia coli* as a fusion protein with maltose-binding protein (MBP-Rep68Δ) which has previously been shown to possess DNA-DNA helicase activity, as does purified wild-type Rep68 (10, 17, 18). We have demonstrated that MBP-Rep68Δ can unwind a DNA-RNA substrate as efficiently

TABLE 1. Oligonucleotides used in helicase assays

Oligonucleotide of substrate	Description	Nucleotide sequence(s)
Oligonucleotide 1	34-mer RNA complementary to M13mp18	$^{5'}$ACCAAUCAAUAAUCGGCUGUCUUUCCUUAUCAUU$^{3'}$
Oligonucleotide 2	34-mer DNA complementary to M13mp18	$^{5'}$ACCAATCAATAATCGGCTGTCTTTCCTTATCATT$^{3'}$
Blunt-ended DNA-RNA substrate		$^{5'}$ACCAAUCAAUAAUCGGCUGUCUUUCCUUAUCAUU$^{3'}$ $^{3'}$TGGTTAGTTATTAGCCGACAGAAAGGAATAGTAA$_{5'}$
RNA-RNA substrate 1	With 5′ overhangs	$^{5'}$N$_{43}$AACUAGUGGAUCCCCC $^{3'}$ $^{3'}$ UUGAUCACCUAGGGGGN$_{73}$ $^{5'}$
RNA-RNA substrate 2	With 3′ overhangs	$^{5'}$ GGGAACAAAAGCUGGCCAAUUCGCCCN$_{113}$$^{3'}$ $^{3'}$N$_{203}$CCCUUGUUUUCGACCGGUUAAGCGGG $_{5'}$

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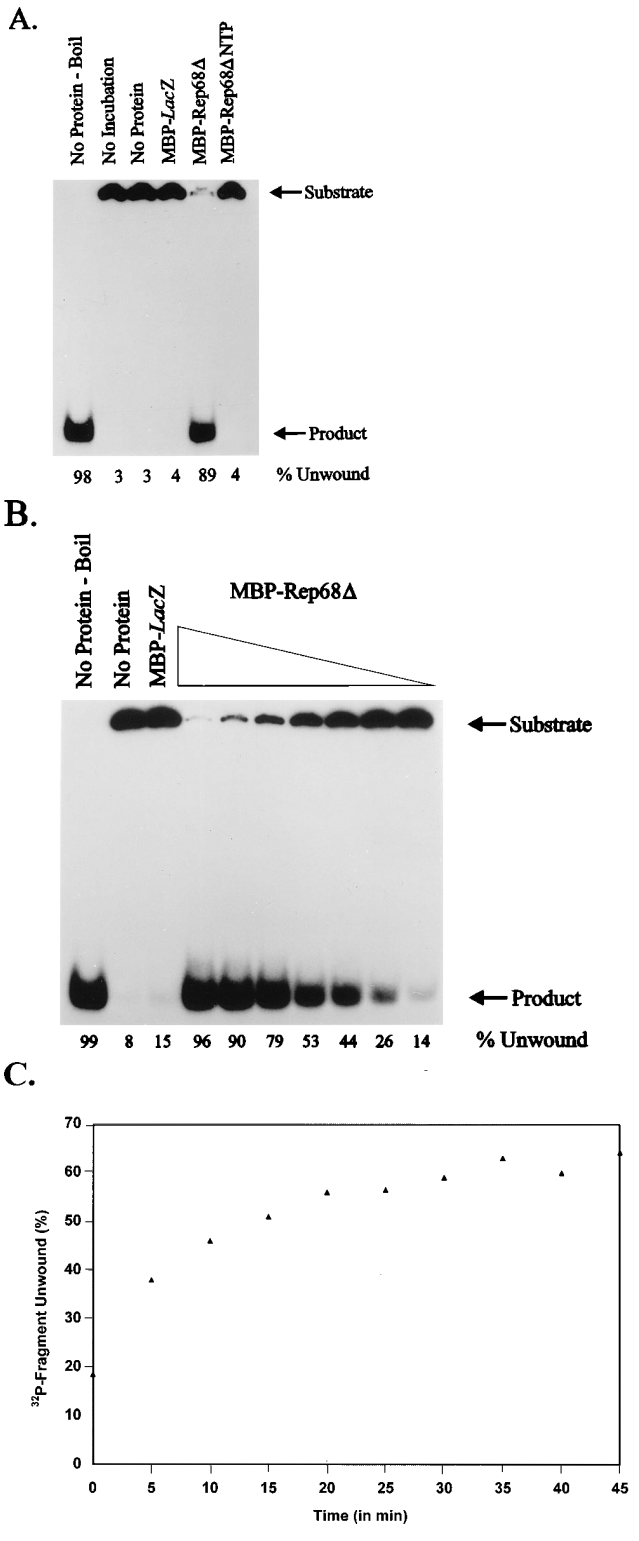

FIG. 1. MBP-Rep68Δ unwinds a DNA-RNA substrate. (A) Helicase assay performed with the fusion proteins indicated above the lanes, 25 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, 20 mM NaCl, 1 mM dithiothreitol, 2 μg of tRNA, 0.4 mM ATP, 0.2 μg of BSA and 5 fmol of the substrate. All reaction mixtures were incubated at 24°C for 35 min. The reaction mixtures for the lanes marked "Boil" were heated to 100°C for 5 min. The position of the substrate and position and percentage of the released product are indicated (arrows). (B) Dose-dependent unwinding of DNA-RNA substrate by MBP-Rep68Δ. Titration was performed with decreasing amounts of MBP-Rep68Δ (4, 2, 1, 0.5, 0.25, 0.12, and 0.06 μg) or 2 μg of MBP-LacZ with 5 fmol of the substrate. (C) Kinetics of MBP-Rep68Δ-mediated DNA-RNA helicase activity. Standard helicase assays were performed for different time intervals, with 1 μg of MBP-Rep68Δ. The percentage of ^{32}P-labeled fragment unwound was calculated by cutting and counting parts of the gel containing the annealed and released oligonucleotide. The data plotted are from a representative experiment.

as it can unwind a DNA-DNA substrate. However, MBP-Rep68Δ lacked the ability to unwind a blunt-ended DNA-RNA substrate or RNA-RNA duplexes. MBP-Rep68Δ also exhibited ATPase activity which was enhanced by the presence of single-stranded DNA. The ability of Rep proteins to unwind DNA-RNA hybrids may play a role in Rep-mediated gene regulation.

MATERIALS AND METHODS

Plasmids and proteins. Plasmid pMAL-c2, which encodes an MBP–β-galactosidase fusion protein (MBP-LacZ) was purchased from New England Biolabs (Beverly, Mass.). Plasmids pMBP-Rep68Δ and pMBP-Rep68ΔNTP, which encode the fusion proteins MBP-Rep68Δ and MBP-Rep68ΔNTP, respectively, have been described previously (10). MBP-Rep68Δ contains within it the same amino acid sequence as wild-type Rep68 except for deletions of 2 amino acids at the N terminus and 16 amino acids at the C terminus. MBP-Rep68ΔNTP is the same as MBP-Rep68Δ except for a lysine-to-histidine substitution at Rep68 amino acid 340. *E. coli* DH5α cells (Life Technologies, Inc., Gaithersburg, Md.) were transformed with these plasmids. The open reading frames for the fusion proteins are under the control of the P_{tac} promoter, and expression can be induced by isopropyl-β-D-thiogalactopyranoside (IPTG). The fusion proteins were isolated by using an amylose resin column (New England Biolabs) and following the protocol recommended by the manufacturer. In brief, the bacterial pellet from a 1-liter culture was resuspended in 50 ml of column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride). The bacteria were lysed by sonication with four 30-s pulses. The suspension was cleared by centrifugation at 9,000 × *g* for 20 min at 4°C. The supernatant was loaded onto a column packed with amylose resin (15- to 20-ml bed volume) equilibrated in column buffer. The column was extensively washed with approximately 8 column volumes of column buffer. The adsorbed proteins were eluted with column buffer containing 10 mM maltose. Fractions were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the production of proteins of the predicted molecular weights. MBP-LacZ, MBP-Rep68Δ, and MBP-Rep68ΔNTP were the predominant species within their respective preparations, as determined by SDS-PAGE and staining with Coomassie blue.

Helicase assay substrates. The DNA-DNA substrate was prepared as follows. A 34-mer oligonucleotide DNA complementary to M13mp18 (Table 1, oligonucleotide 2) was synthesized with an Applied Biosystems (Foster City, Calif.) 381A DNA synthesizer. It was radiolabeled with polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (Dupont NEN, Boston, Mass.) according to the protocol recommended by the manufacturer and was annealed to M13mp18 viral DNA (single-stranded circular template) (47). After the annealing, the reaction mixture was passed through a Sepharose CL-2B (Pharmacia, Piscataway, N.J.) column to separate the annealed oligomer from unannealed oligomer and unincorporated nucleotides.

The standard DNA-RNA substrate was prepared as follows. A 34-mer oligonucleotide RNA was synthesized with an Applied Biosystems 380B DNA/RNA synthesizer (Table 1, oligonucleotide 1, which is complementary to the same region of M13mp18 as oligonucleotide 2). It was end labeled and annealed to single-stranded M13mp18 viral DNA. After the annealing, the substrate was purified by gel filtration chromatography as described above for the DNA-DNA substrate.

The blunt-ended DNA-RNA substrate was prepared by annealing an end-labeled 34-mer RNA to a complementary end-labeled 34-mer DNA. The annealed substrate was purified by electrophoresis in a 15% nondenaturing polyacrylamide gel. The radiolabeled substrate was located by autoradiography and eluted from crushed gel pieces into 0.5 M ammonium acetate–0.1% (wt/vol) SDS at room temperature overnight. The eluate was extracted with phenol-chloroform, and the nucleic acid was precipitated with ethanol.

RNA-RNA substrate 1 (with 5′ overhangs) was produced by using the plasmid pBluescript II SK(+) (Stratagene, La Jolla, Calif.), which contains the T3 and T7 promoters on either side of a multiple cloning sequence. The plasmid linearized with either *Sma*I or *Xba*I was used as the template to produce transcripts in vitro with either T3 or T7 polymerase, respectively, according to the instructions supplied by the manufacturer (Promega, Madison, Wis.). The transcription reaction mixtures contained [α-^{32}P]CTP. The T3 and the T7 transcripts were 59 and 89 nucleotides (nt) long, respectively, and contained 16 nt of overlapping sequence.

To produce RNA-RNA substrate 2 (with 3′ overhangs), plasmid pBluescript II

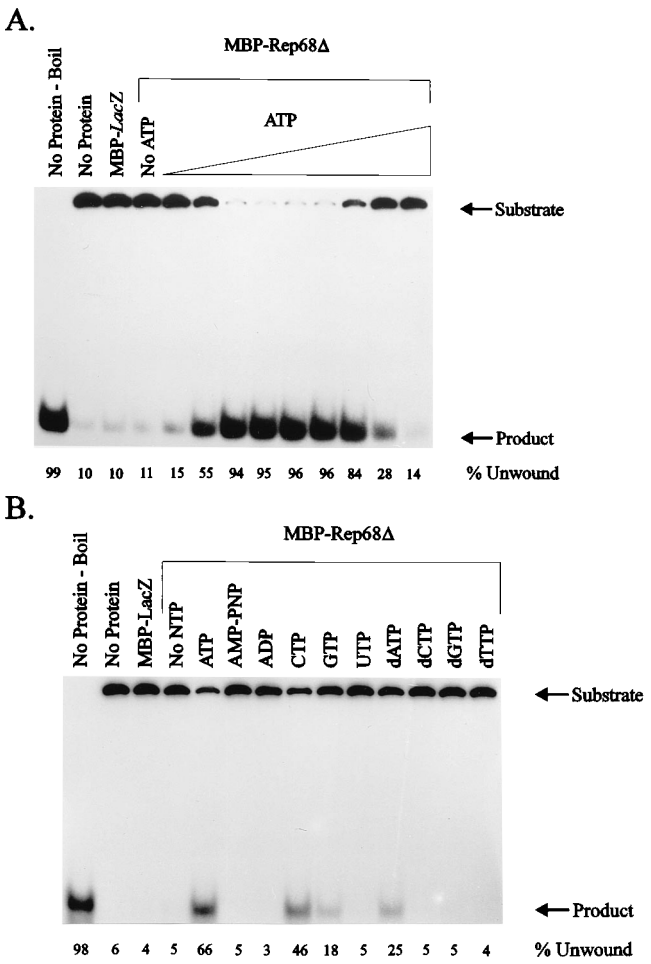

FIG. 2. Characterization of MBP-Rep68Δ helicase activity. Each reaction mixture contained 5 fmol of the substrate, 2 μg of fusion protein, and all other constituents for the standard helicase assay (as detailed in Materials and Methods). The reaction mixture for the lanes marked "Boil" was heated to 100°C for 5 min. (A) Effects of increasing concentrations of ATP (0.06, 0.12, 0.5, 1, 2, 4, 8, 16, and 32 mM) on MBP-Rep68Δ-mediated DNA-RNA helicase activity. (B) Effects of different NTPs, dNTPs, ADP, and 5′ adenylyl-imidodiphosphate (all at 0.1 mM) on MBP-Rep68Δ-mediated DNA-RNA helicase activity. The reaction mixtures for lanes denoted "No protein" and "MBP-LacZ" contained 0.4 mM ATP. The position of the substrate and position and percentage of the released product are indicated (arrows).

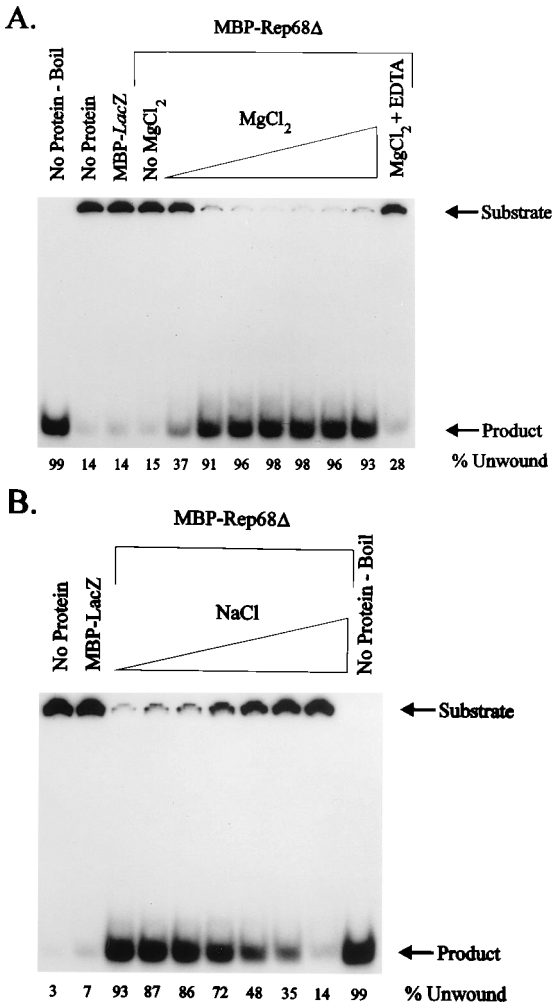

FIG. 3. Effects of different salts on MBP-Rep68Δ-mediated helicase activity. Helicase assays were performed with 2 μg of fusion protein and 5 fmol of the substrate. The reaction mixture for the lanes marked "Boil" was heated to 100°C for 5 min. The position of the substrate and position and percentage of the released product are indicated (arrows). (A) Effects of increasing concentrations of $MgCl_2$ (0.08, 0.15, 0.3, 0.6, 1.3, 2.5, and 5 mM) on MBP-Rep68Δ-mediated helicase activity. The $MgCl_2$ + EDTA lane contained 5 mM $MgCl_2$ plus 10 mM EDTA. (B) Effects of increasing concentrations of NaCl (28, 36, 51, 83, 145, and 270 mM) on MBP-Rep68Δ-mediated DNA-RNA helicase activity.

SK(+) was cut with *Sac*I and *Kpn*I, the overhangs were blunted by T4 DNA polymerase, and the blunt ends were ligated with T4 DNA ligase. The resulting plasmid was cut with *Pvu*II to yield a 438-bp fragment which was used as the template for in vitro transcription from the T3 and T7 promoters. The T3 and T7 transcripts were 139 and 229 nt long, respectively, and contained 26 nt of overlapping sequence.

After the in vitro transcription, the reaction mixtures were extracted with phenol-chloroform and the nucleic acids were precipitated by ethanol, redissolved in TBE buffer (89 mM Tris-borate [pH 8.3], 2 mM EDTA) containing 90% formamide, and heated at 95°C for 3 min. Samples were electrophoresed in 6% polyacrylamide gels made in TBE buffer and containing 8 M urea. The radiolabeled transcripts were located by autoradiography and were eluted from crushed gel pieces into 0.5 M ammonium acetate–0.1% (wt/vol) SDS at room temperature overnight. The eluates were extracted with phenol-chloroform, and the RNA was precipitated with ethanol. The RNA-RNA substrates were prepared by mixing equal picomole quantities of ^{32}P-labeled T3 and T7 transcripts in buffer containing 10 mM Tris-HCl (pH 7.5)–0.1 mM EDTA–200 mM NaCl prior to heating at 95°C for 5 min and at 67°C for 1 h followed by a slow cooling to room temperature. The annealed substrates were purified by electrophoresis in a 6% nondenaturing polyacrylamide gel. The radiolabeled substrates were located by autoradiography and were eluted from crushed gel pieces into 0.5 M ammonium acetate–0.1% (wt/vol) SDS at room temperature overnight. The eluates were extracted with phenol-chloroform, and the RNA was precipitated with ethanol.

Helicase assay. The standard conditions for the helicase assay were essentially the same as those described by Im and Muzyczka (17, 18). Approximately 5 fmol of annealed substrate was added to 20 μl of a mixture that contained 25 mM 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid) (HEPES)–KOH (pH 7.5), 5 mM $MgCl_2$, 20 mM NaCl, 1 mM dithiothreitol, 2 μg of tRNA, 0.4 mM ATP, 0.2 μg of bovine serum albumin (BSA), and various amounts of the fusion proteins. The reaction mixture was incubated at 24°C for 35 min. The reaction was terminated by the addition of 10 μl of helicase loading buffer (0.5% SDS, 50 mM EDTA, 40% [vol/vol] glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol). The reaction products were fractionated on a nondenaturing 8% polyacrylamide gel. The gel was then dried and exposed to X-ray film for autoradiography. The autoradiograph was aligned with the dried gel, the substrate and product bands were cut out, and the radioactivity associated with the gel pieces was quantitated with a liquid scintillation counter (Beckman, Fullerton, Calif.).

ATPase assay. ATPase assays were carried out according to the protocol described by Warrener et al. (45) in a final volume of 10 μl containing 50 mM Tris-HCl (pH 8.0), 20 or 50 mM NaCl, 2.5 mM $MgCl_2$, 2 nmol of [α-^{32}P]ATP (3,000 Ci/mmol), and 0.5 or 2 μg of MBP fusion protein. For some reactions, 0.2 μg of either single-stranded M13mp18 DNA, poly(A), or poly(U) was added. The reaction mixtures were incubated at 24°C for 60 min, and then the reactions were terminated by the addition of EDTA to a final concentration of 20 mM. The

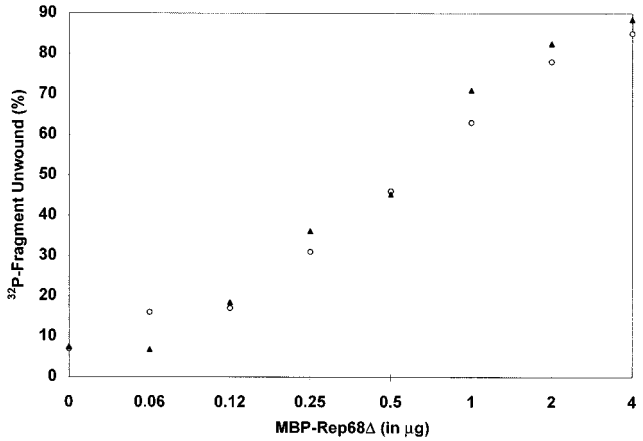

FIG. 4. Comparison of DNA-DNA and DNA-RNA helicase activities of MBP-Rep68Δ. Helicase assays were performed with various amounts of MBP-Rep68Δ and 5 fmol of either the DNA-DNA substrate (open circles) or DNA-RNA substrate (closed triangles). The gel pieces containing the substrate and the released ^{32}P-labeled oligonucleotide were cut and counted, and the percentage unwound was determined. The data plotted are from a representative experiment.

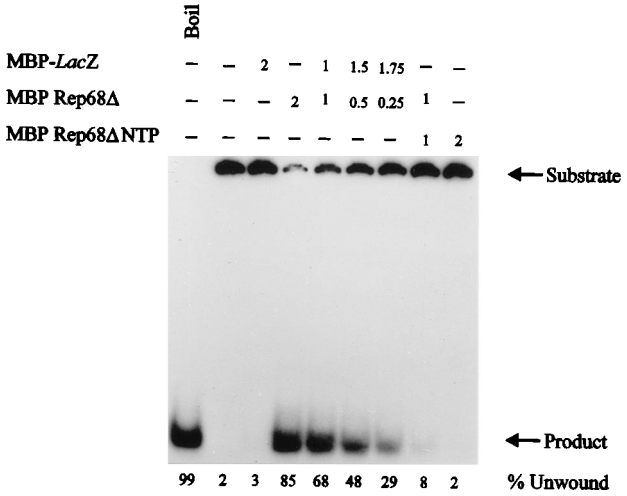

FIG. 5. Inhibition of MBP-Rep68Δ-mediated DNA-RNA helicase activity by MBP-Rep68ΔNTP. The amounts (in micrograms) of fusion protein in each standard reaction mixture are indicated above the lanes. Helicase assays were performed with 5 fmol of the substrate. The reaction mixture for the lane marked "Boil" was heated to 100°C for 5 min. The position of the substrate and position and percentage of the released product are indicated (arrows).

reaction products were analyzed by thin-layer chromatography. Two microliters of the reaction mixture was spotted onto plastic-backed polyethyleneimine-cellulose sheets (E M Science, Gibbstown, N.J.) and developed by ascending chromatography in 0.375 M potassium phosphate (pH 3.5). The sheets were dried and exposed to X-ray film. The spots containing the radioactive substrate and product were then scraped, and the radioactivity was quantitated with a liquid scintillation counter.

RESULTS

MBP-Rep68Δ unwinds DNA-RNA substrate. It has been shown previously that wild-type Rep68 and MBP-Rep68Δ exhibit DNA-DNA helicase activity (10, 17, 18). In this study, the ability of MBP-Rep68Δ to unwind DNA-RNA hybrids was tested by using a 34-nt RNA annealed to a complementary region of M13mp18 as the substrate. Incubation of the DNA-RNA substrate with MBP-Rep68Δ in the presence of Mg^{2+} and ATP resulted in the displacement of the radiolabeled RNA, as analyzed by PAGE followed by autoradiography (Fig. 1A). However, MBP-LacZ, and MBP-Rep68ΔNTP did not unwind the substrate. There was decreased unwinding of the substrate with a decrease in the amount of MBP-Rep68Δ in the reaction mixture (Fig. 1B). The kinetic studies indicated a continuous increase in the amount of substrate unwound for the first 35 min (Fig. 1C).

Characterization of MBP-Rep68Δ-mediated DNA-RNA helicase activity. Many helicases possess nucleoside triphosphatase (NTPase) activity, which is often activated when the helicase binds to single-stranded nucleic acid (27). Binding of nucleoside triphosphates (NTPs) to the helicase and the subsequent hydrolysis of NTPs provides the energy required for the translocation of the helicase enzyme along the bound nucleic acid and for the denaturation of the duplex region (22, 27). As seen from Fig. 2A, MBP-Rep68Δ did not unwind the DNA-RNA substrate in the absence of ATP. There was an increase in MBP-Rep-mediated helicase activity as the concentration of ATP in the reaction mixture was increased. However, concentrations higher than 4 mM inhibited the DNA-RNA unwinding activity of MBP-Rep68Δ. There was very little, if any, unwinding activity in the presence of ADP or 5′ adenylyl-imidodiphosphate (a nonhydrolyzable analog of ATP). At a 0.1 mM concentration of NTPs, MBP-Rep68Δ-mediated helicase activity seemed to prefer ATP to the other NTPs or dNTPs. The order of preference was ATP > CTP > dATP > GTP > UTP = dCTP = dGTP = dTTP (Fig. 2B). The order of preference for the different NTPs in MBP-Rep68Δ-mediated helicase activity is consistent with the order of NTP preference in Rep68-mediated endonuclease activity observed by Im and Muzyczka (18).

Another important requirement of most helicases is the presence of divalent cations. Very little DNA-RNA helicase activity occurred in the absence of $MgCl_2$ or in the presence of $MgCl_2$ plus EDTA. An $MgCl_2$ concentration of at least 75 μM was required for detectable helicase activity (Fig. 3A). Since ionic strength is known to affect the enzymatic activities of proteins, we examined the effects of various concentrations of NaCl on the DNA-RNA helicase activity of MBP-Rep68Δ. As seen in Fig. 3B, NaCl concentrations of 36 mM and above inhibited DNA-RNA helicase activity. A pH of 6.6 or lower inhibited DNA-RNA helicase activity (data not shown).

Comparison of DNA-DNA and DNA-RNA helicase activities of MBP-Rep68Δ. To compare the efficiency of MBP-Rep68Δ in unwinding DNA-DNA and DNA-RNA substrates, we annealed either a 34-mer RNA (Table 1, oligonucleotide 1) or a 34-mer DNA (Table 1, oligonucleotide 2) to single-stranded M13mp18 viral DNA (both oligonucleotides were complementary to the same region of M13). Equal picomole quantities of DNA-DNA and DNA-RNA substrates were added to the reaction mixtures, and the efficiencies of unwinding were compared. MBP-Rep68Δ unwound DNA-DNA and DNA-RNA substrates with almost equal efficiency (Fig. 4).

MBP-Rep68ΔNTP inhibits the DNA-RNA helicase activity of MBP-Rep68Δ. The *trans*-dominant inhibitory effect of Rep proteins with a K340H mutation on the activities of wild-type Rep proteins has been shown to occur in vitro with respect to AAV terminal resolution (33, 35), *trs* endonuclease activity (35), and DNA-DNA helicase activity (34) and in vivo in AAV replication (9). In this study, we investigated this *trans*-dominant inhibitory effect of MBP-Rep68ΔNTP on the DNA-RNA helicase activity of MBP-Rep68Δ. As shown by Fig. 5, when MBP-Rep68Δ and MBP-Rep68ΔNTP were mixed at a 1:1 ra-

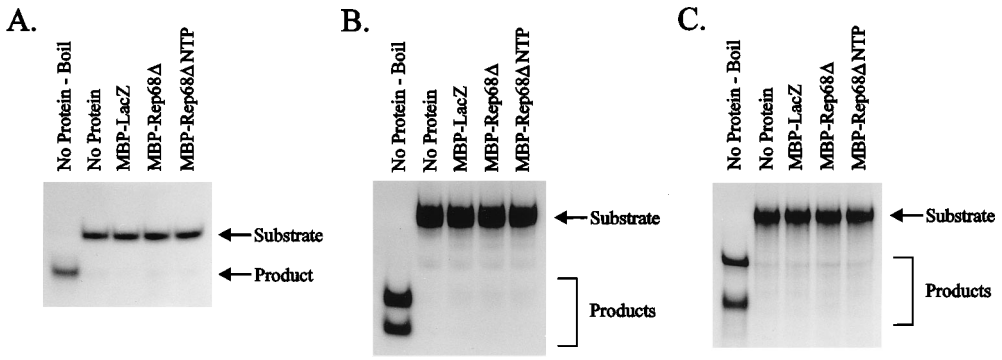

FIG. 6. MBP-Rep68Δ cannot unwind blunt-ended DNA-RNA or duplex RNA substrates. Helicase assays were performed with 2 μg of fusion protein and 5 fmol of the substrate. (A) MBP-Rep68Δ does not unwind a blunt-ended DNA-RNA substrate (sequence shown in Table 1). (B) MBP-Rep68Δ does not unwind an RNA-RNA substrate with 5′ overhangs (Table 1, RNA-RNA substrate 1). (C) MBP-Rep68Δ does not unwind an RNA-RNA substrate with 3′ overhangs (Table 1, RNA-RNA substrate 2). The reaction mixtures for the lanes marked "Boil" were heated to 100°C for 5 min. The positions of the substrate and the products are indicated (arrows). Substrates were made as described in Materials and Methods.

tio, MBP-Rep68ΔNTP inhibited the DNA-RNA helicase activity of MBP-Rep68Δ by more than fourfold.

MBP-Rep68Δ does not unwind blunt-ended DNA-RNA or duplex RNA substrates. To investigate whether MBP-Rep68Δ requires single-stranded regions to bind and translocate before it encounters and unwinds DNA-RNA duplex regions, we constructed a blunt-ended DNA-RNA substrate as shown in Table 1. MBP-Rep68Δ did not unwind this blunt-ended RNA-DNA substrate, though the reaction conditions optimum for DNA-RNA helicase activity were provided (Fig. 6A). This result indicated that the MBP-Rep68Δ-mediated DNA-RNA helicase activity requires stretches of single-stranded nucleic acid to which the protein can bind and translocate before unwinding double-stranded regions.

The ability of MBP-Rep68Δ to unwind RNA-RNA hybrids was also tested. Duplex RNA substrates (Table 1) were prepared by hybridizing partially complementary RNA strands obtained from in vitro transcription reactions. MBP-Rep68Δ did not unwind RNA duplexes with either 3′ or 5′ overhangs (Fig. 6B and C). This result suggests that MBP-Rep68Δ requires single-stranded DNA specifically to translocate before it can unwind duplex regions and that single-stranded RNA regions cannot substitute for single-stranded DNA.

MBP-Rep68Δ has ATPase activity. Though it has been presumed that AAV Rep proteins have ATPase activity, it has never been demonstrated. We have shown here for the first time that MBP-Rep68Δ has ATPase activity (Fig. 7A), since MBP-Rep68Δ hydrolyzed ATP to a compound that comigrates with ADP. MBP-Rep68Δ-mediated ATPase activity was enhanced at least twofold by the presence of single-stranded M13 DNA but not by RNA polymers like poly(A) and poly(U) (Fig. 7B). MBP-Rep68Δ-mediated ATPase activity required the presence of Mg^{2+} ions, since very little, if any, ATPase activity occurred in the absence of $MgCl_2$ or in the presence of $MgCl_2$ plus EDTA (Fig. 7A).

MBP-Rep68ΔNTP has a mutation in the NTP-binding site (9, 10) which changes the codon for lysine 340 of Rep68 to a histidine codon (K340H). This mutant has been previously shown to lack DNA-DNA helicase activity (10). We have shown that this mutant also lacks DNA-RNA helicase activity (Fig. 1A). The inability of this mutant to unwind DNA-DNA or DNA-RNA may be due to its inability to hydrolyze ATP. This hypothesis was tested by studying the ATPase activities of MBP-Rep68Δ and MBP-Rep68ΔNTP. The results from the ATPase assays indicated that MBP-Rep68ΔNTP exhibited no significant ATPase activity (Fig. 7A).

DISCUSSION

Rep proteins of AAV have been shown to possess ATP-dependent DNA-DNA helicase and endonuclease activities and to play an important role in the replication and gene expression of the virus. The creation of plasmid pMBP-Rep68Δ has allowed the production of large quantities of relatively pure Rep fusion protein for functional analysis (10). In spite of the presence of the maltose-binding domain, MBP-Rep68Δ has performed comparably to wild-type Rep68 in all previous in vitro assays (10, 44). In this study, we have shown that MBP-Rep68Δ also exhibits DNA-RNA helicase activity which is comparable to its DNA-DNA helicase activity. We have also shown for the first time that MBP-Rep68Δ hydrolyzes ATP to ADP. MBP-Rep68ΔNTP, which lacks DNA-RNA helicase activity, also lacks ATPase activity, indicating that ATP hydrolysis and unwinding of the DNA-RNA hybrid are associated.

The helicases of parvoviruses have sequence homology with the superfamily III of ATPases and have been suspected to possess ATPase activities (21). They contain three conserved sequence motifs (called motifs A, B, and C) packed in an approximately 100-amino-acid domain (11). Motif A (the so-called P loop) has been shown to directly interact with the phosphate moiety of the NTP substrate in several ATPases and GTPases (37, 39). The lysine residue, which has been mutated to histidine in MBP-Rep68ΔNTP, is a conserved residue in motif A. The inability of MBP-Rep68ΔNTP to hydrolyze ATP establishes the involvement of the conserved lysine in ATP hydrolysis. However, Jindal et al. (19) have demonstrated that mutation of the conserved lysine residue (in motif A) to serine did not abolish the ATPase activity of the analogous NS-1 protein of minute virus of mice (an autonomous parvovirus).

Mixing experiments showed that MBP-Rep68ΔNTP is dominant negative for DNA-RNA helicase activity. Greater than fourfold inhibition was observed when MBP-Rep68ΔNTP and MBP-Rep68Δ were mixed in a 1:1 ratio. Previous studies have revealed that the K340H mutant of Rep78 can inhibit both the AAV terminal resolution and terminal resolution site endonuclease activities of the wild-type protein (33, 35). Human cell nuclear extracts containing Rep78 protein with the K340H mutation also inhibited the DNA-DNA helicase activity of nuclear extracts containing wild-type Rep68 protein (34). These observations are consistent with the hypothesis that the functional forms of Rep68 and Rep78 are multimers and that a defective subunit could inactivate the entire complex.

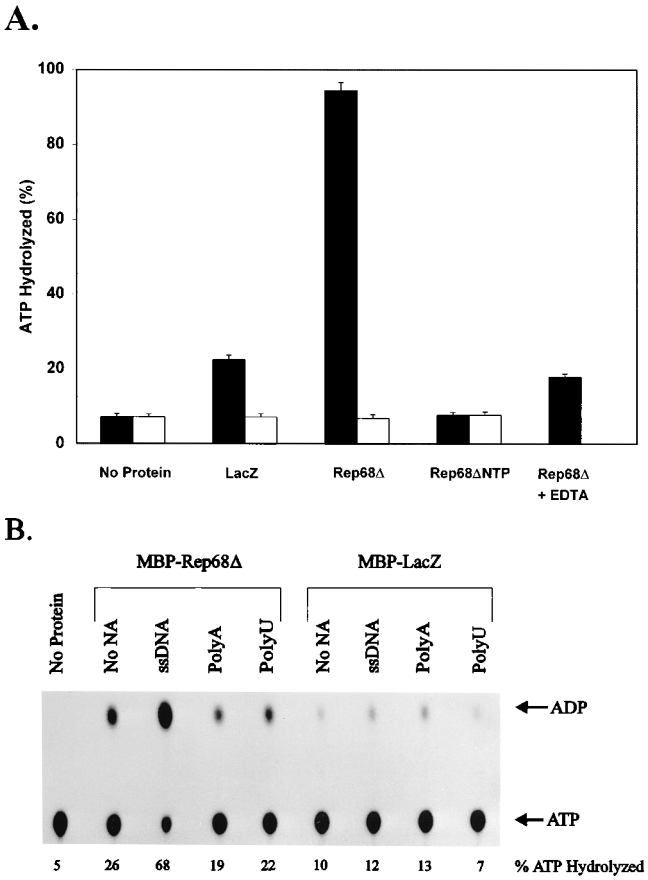

FIG. 7. Characterization of MBP-Rep68Δ ATPase activity. Standard ATPase assays (as described in Materials and Methods) were performed with 2 nmol of [α-^{32}P]ATP, and the percentage of ATP hydrolyzed was quantitated. LacZ, MBP-LacZ; Rep68Δ, MBP-Rep68Δ; Rep68ΔNTP, MBP-Rep68ΔNTP. (A) Comparison of ATPase activities of MBP-Rep68Δ and MBP-Rep68ΔNTP and the effect of Mg^{2+} ions on MBP-Rep68Δ-mediated ATPase activity. The assays were carried out in the presence (closed bars) or the absence (open bars) of 2.5 mM $MgCl_2$. Each reaction mixture contained 2 μg of MBP fusion protein, 50 mM NaCl, and 0.2 μg of M13mp18 single-stranded viral DNA. The bar labeled Rep68Δ+EDTA denotes reactions carried out in the presence of 2 μg of MBP-Rep68Δ, 2.5 mM $MgCl_2$, and 20 mM EDTA. Error bars represent 1 standard deviation from at least three trials. (B) Effects of nucleic acids on MBP-Rep68Δ-mediated ATPase activity. Each reaction mixture contained 0.5 μg of MBP fusion protein and 20 mM NaCl. Reactions carried out in the absence of nucleic acids (No NA) and assays carried out in the presence of 0.2 μg of either M13mp18 single-stranded viral DNA (ssDNA) or RNA polymers poly(A) (PolyA) and poly(U) (PolyU) are indicated. The percentage of ATP hydrolyzed is indicated below each lane.

MBP-Rep68Δ-mediated DNA-RNA helicase activity is inhibited by concentrations of NaCl above 36 mM. This inhibition is probably due to the stabilization of hybridization within the substrate. A similar phenomenon was noticed with *E. coli* Rho protein. The efficiency of unwinding of DNA-RNA hybrids by Rho was increased 20-fold by decreasing the KCl concentration in the reaction mixture (5).

Not all DNA-DNA helicases can unwind DNA-RNA hybrids. Some helicases can unwind only DNA duplexes (e.g., Rep, helicase I, and helicase IV proteins of *E. coli*) (26, 27), while others unwind DNA duplexes as well as DNA-RNA hybrids (e.g., UvrD protein of *E. coli* and RAD3 protein of *Saccharomyces cerevisiae*). A few helicases like the simian virus 40 T antigen are capable of unwinding DNA duplexes, RNA duplexes, and DNA-RNA hybrids (38, 43). There are also proteins, like the human p68 protein, that specifically unwind RNA duplexes (15). p68 does not unwind duplex DNA, and its ATPase activity is stimulated by RNA and not by single-stranded DNA. In contrast, the ATPase activity of MBP-Rep68Δ was enhanced by single-stranded DNA. In addition, MBP-Rep68Δ could not unwind blunt-ended DNA-RNA hybrids or duplex RNA. Taken together, these observations strongly suggest that MBP-Rep68Δ requires stretches of single-stranded DNA to bind and translocate upon before it encounters the duplex region and that binding to a single-stranded region of DNA in some way stimulates its ATPase activity, which in turn might provide energy for unwinding. This theory also explains why RNA could not enhance the ATPase activity of MBP-Rep68Δ.

DNA-RNA helicase activity may be important in transcription and other processes of the cell. For example, the *E. coli* Rho protein has been shown to catalyze the unwinding of specific RNA-DNA hybrids in an ATP-dependent reaction (4). It is speculated that Rho-mediated transcription termination may occur by virtue of its DNA-RNA helicase activity. AAV Rep proteins may also affect transcription by unwinding DNA-RNA hybrids. During transcription, there is an unwinding of double-stranded DNA to expose single-stranded regions and the anticoding strand of DNA is used as the template to synthesize mRNA. Rep68, by virtue of its DNA-RNA helicase activity, may displace nascent mRNA from its template and thereby block the production of full-length transcripts. Rep78 and Rep68 have been shown to negatively regulate mRNA levels from the p_5 and p_{19} promoters of AAV in vivo (3, 23). There seems to be a significant difference in the mechanisms of regulation of p_5 and p_{19} transcript levels by Rep proteins. Rep78/K340H, which has the same mutation in the putative NTP-binding site as MBP-Rep68ΔNTP, inhibited expression from the p_5 promoter but not from the p_{19} promoter (23). Thus, an ATP-dependent function is suspected in Rep-mediated down-regulation of p_{19} transcripts.

It has been shown that AAV inhibits replication of HIV-1, the causative agent of AIDS (29). There is also experimental evidence that Rep68 and Rep78 are sufficient for mediating the inhibition (1, 32, 36), while the NTP mutant (K340H) is not (36). Though there have been several reports indicating the involvement of AAV Rep proteins in the inhibition of HIV production, the mechanism by which AAV Rep proteins inhibit the HIV life cycle has not yet been clearly elucidated. The DNA-RNA helicase activity of Rep68 may block HIV-1 transcription by a mechanism similar to the one that we have proposed for p_{19}.

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