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Bluetongue Virus VP6 Protein Binds ATP and Exhibits an RNA-Dependent ATPase Function and a Helicase Activity That Catalyze the Unwinding of Double-Stranded RNA Substrates

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RNA-dependent ATPase and helicase activities have been identified associated with the purified VP6 protein of bluetongue virus, a member of the *Orbivirus* genus of double-stranded RNA (dsRNA; *Reoviridae* family) viruses. In addition, the protein has an ATP binding activity. RNA unwinding of duplexes occurred with both 3′ and 5′ overhang templates, as well as with blunt-ended dsRNA, an activity not previously identified in other viral helicases. Although little sequence similarity to other helicases was detected, certain similarities to motifs commonly attributed to such proteins were identified.

Helicases catalyze the unwinding of double-stranded nucleic acids, thereby allowing the initiation of replication and transcription. With an ATPase activity to provide energy, the proteins may exhibit either DNA- or RNA-unwinding activities or both (2, 4, 21, 45, 48). A number of helicases have been described for viruses, including both DNA and RNA viruses (7, 9, 19, 24, 29, 47, 49, 52, 55). Sequence comparisons of helicases have identified various common protein motifs, including putative sequences for ATP binding and helicase functions (1, 12, 13, 22, 27). In this study we report the presence of both helicase and ATPase activities associated with the purified VP6 protein of bluetongue virus (BTV), a double-stranded RNA (dsRNA) genome virus (*Reoviridae* family), and present evidence concerning the characteristics of the enzyme activities.

While a number of properties of orbiviruses are distinct by comparison to other members of the *Reoviridae* family (e.g., orbiviruses replicate in both vertebrate and invertebrate hosts [40]), like reoviruses and rotaviruses, orbiviruses transcribe mRNA from the various segments of the viral dsRNA genome. However, exactly how transcription occurs is not known. For BTV, upon entry into a permissive cell the viral outer shell proteins are modified and/or removed from the infecting virion, and the viral core is released into the cytoplasm where it becomes transcriptionally active. In agreement with this view, mRNA synthesis can be activated in vitro by proteolytic removal of the outer capsid proteins VP2 and VP5 from virus particles (34). The icosahedral core of BTV consists of 10 dsRNA segments, three minor proteins (VP1, VP4, and VP6), and two major structural proteins, VP3 and VP7. Transcription occurs inside the viral core and involves the extrusion of capped and methylated mRNA species (35) that are subsequently translated into viral proteins in the cytoplasm of an infected cell. Newly produced viral proteins later interact with sequestered viral mRNA species in so-called viral inclusion bodies to form proviral particles, which are the sites for dsRNA synthesis and the further production of mRNA prior to eventual formation of complete virus particles and extrusion and release from an infected cell (17).

The three minor core proteins of BTV are all thought to be associated with the processes of transcription and replication, although their specific functions are understood only in general terms. VP4 has been shown to be the mRNA capping enzyme (30), while VP1 is the RNA-dependent RNA polymerase (50). The third minor core protein, VP6, is encoded by BTV segment 9 (S9) dsRNA and is composed of 328 amino acids giving an M_r of 35,750. The protein has a high proportion of hydrophilic amino acids and contains the highest percentage of positively charged amino acids among all the BTV-encoded proteins. It is also rich in glycine. A baculovirus-expressed BTV VP6 has been demonstrated to bind single-stranded RNA (ssRNA), as well as dsRNA and DNA (41), and it has been suggested to function as a viral helicase (39). By deletion analyses the nucleic acid-binding domains of VP6 have been partially localized (15). In the present investigation recombinant VP6 of BTV serotype 10 (BTV-10) was purified and used to demonstrate ATP binding and RNA-dependent ATP hydrolysis and helicase functions with a number of RNA substrates.

MATERIALS AND METHODS

Expression and purification of BTV-10 VP6. *Spodoptera frugiperda* Sf9 insect cells were infected with recombinant AcBTV10-9 baculovirus (41) at a multiplicity of infection of 5 PFU/cell and incubated in TC100 medium containing 10% fetal calf serum for 72 h at 28°C. The cells were harvested by centrifugation at 4,000 × *g* for 10 min, rinsed in phosphate-buffered saline, and resuspended at 2.5×10^7 cells/ml in TENT buffer (100 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 1% [vol/vol] Triton X-100, pH 7.4) in the presence of 1 mM dithiothreitol (DTT) and a cocktail of protease inhibitors comprising 10 μM APMSF, 10 μM E-64, 10 μM leupeptin, and 1 μM pepstatin A (final concentrations). Infected cells were lysed with 10 strokes of a Dounce homogenizer and incubated on ice for 10 min. A sample was taken (Fig. 1, lane 1), and then the cell debris and nuclei was removed by centrifugation at 4,000 × *g* for 10 min. The resulting cell supernatant (Fig. 1, lane 2) was first adjusted to 40% saturated ammonium sulfate and then centrifuged at 40,000 × *g* for 20 min. The resulting supernatant

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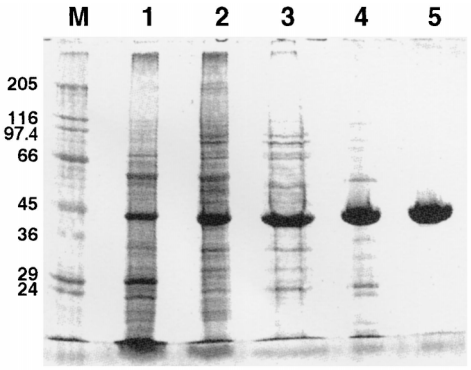

FIG. 1. Purification of recombinant BTV-10 VP6 from extracts of baculovirus-infected Sf9 insect cells. Protein samples were resolved by SDS-PAGE prior to being stained with Page Blue G 90 (Fluka). Lane M, protein markers (sizes in kilodaltons are shown at left); lane 1, total cell homogenate; lane 2, cell supernatant after Dounce homogenization and low-speed centrifugation; lane 3, proteins recovered after ammonium sulfate fractionation; lane 4, proteins recovered after phenyl-Sepharose chromatography; lane 5, purified VP6 after Q-Sepharose chromatography.

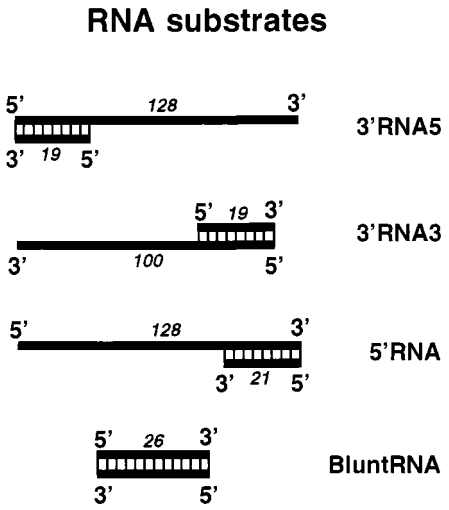

FIG. 2. Schematic representation of the RNA substrates used in the helicase assays. The top strand of each duplex represents the coding strand (positive-sense) sequence, while the bottom strand represents the noncoding strand (negative-sense) sequence of BTV-10 S10 dsRNA. Thin vertical lines are regions of base pairing. The italicized numbers indicate the nucleotide lengths in the duplex portion of the substrate. For details, see Materials and Methods.

was then adjusted to 90% saturated ammonium sulfate. The precipitate obtained after a similar centrifugation was resuspended in a minimal volume of 80 mM sodium phosphate, pH 6.8, and 40% saturated ammonium sulfate (Fig. 1, lane 3). After clarification, this preparation was loaded onto a phenyl-Sepharose CL-4B (Pharmacia) column (internal diameter, 10 mm; bed height, 100 mm) previously equilibrated with 80 mM sodium phosphate, pH 6.8, and 40% ammonium sulfate. Protein was eluted with an ammonium sulfate gradient, 40 to 0% saturation in 80 mM sodium phosphate, pH 6.8. Fractions containing the majority of the VP6 protein were eluted in 20% saturated ammonium sulfate, identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pooled (Fig. 1, lane 4), and concentrated by precipitation with 90% saturated ammonium sulfate. The VP6 protein was then resuspended, and the buffer was exchanged into 20 mM Tris-HCl–20 mM NaCl (pH 8.5) by using a PD-10 column (Pharmacia). Anion ion-exchange chromatography was subsequently performed on the VP6 preparation by using a 1-ml Q-Sepharose HiTrap column (Pharmacia) equilibrated with 20 mM Tris-HCl–20 mM NaCl, pH 8.5. Bound proteins were eluted by using an NaCl gradient (20 to 500 mM in 20 mM Tris-HCl, pH 8.5). Purified BTV-10 VP6 protein was eluted with 70 mM NaCl (Fig. 1, lane 5), fractions were pooled, and the buffer was exchanged into 30 mM Tris-HCl (pH 7.5) by ultrafiltration with Centricon-10 devices (Amicon). The cocktail of protease inhibitors (at the concentrations referred to above) was added to the VP6 samples throughout each stage of the purification scheme. The purified VP6 was at least 99% pure as determined from the Page Blue-stained gel, and subsequent analysis could detect no significant levels of contaminating protein even after silver staining (data not shown).

Preparation of helicase substrates. The dsRNA templates employed in the helicase assays either had two blunt ends (Fig. 2, BluntRNA) or had one end with a 3′ overhang and one blunt end (representing the 5′ end of the coding, positive-sense, strand of the S10 dsRNA) (Fig. 2, 3′RNA5) or possessed one blunt end (representing the 3′ end of the positive-sense strand of the S10 dsRNA) and one end with a 3′ overhang (Fig. 2, 3′RNA3), or had one blunt end and one end with a 5′ overhang (representing the 5′ end of the positive-sense strand) (Fig. 2, 5′RNA). To prepare the 3′RNA5 and 3′RNA3 duplexes, short, labeled ssRNA species, referred to as the release strands (i.e., the labeled RNA released from the derived duplexes by the action of VP6 helicase), were synthesized by annealing a T7 promoter oligonucleotide (5′-GCG TAA TAC GAC TCA CTA TA-3′) to an oligonucleotide with a complementary sequence downstream of a BTV sequence (underlined) representing the complement of the desired BTV product. Thus for the 3′RNA5, the following oligonucleotide template was used to prepare the 19-nucleotide-long, negative-sense, release RNA: 5′-GTT AAA AAG TGT CGC TGC CTA TAG TGA GTC GTA TTA CGC-3′. Likewise, for the 19-nucleotide-long 3′RNA3 positive-sense release strand, the following template was used: 5′-GTA AGT GTG TAG TAT CGC GTA TAG TGA GTC GTA TTA CGC-3′. With these templates and the T7 promoter oligonucleotide, in vitro T7 polymerase-catalyzed RNA transcription assays were undertaken according to the manufacturer's protocol (AmpliScribe T7 transcription kit; Epicentre Technologies, Cambio, Cambridge, United Kingdom) and in the presence of [α-^{32}P]UTP to obtain the desired labeled 19-nucleotide RNAs (Fig. 2, 3′RNA5 and 3′RNA3).

To prepare the 3′RNA5 duplex, the exact-copy vector pEC-10B10-Hδ was linearized with *Sty*I and used for run-off transcription to synthesize a 128-nucleotide-long RNA representing the 5′ end of the positive-sense strand up to an internal *Sty*I site present in the S10 cDNA. The product was combined with the corresponding labeled negative-sense release strand, and the mixture was denatured for 4 min at 95°C by using a 10-fold excess of unlabeled to labeled RNA in 25 mM HEPES buffer (pH 7.5)–400 mM NaCl–1 mM EDTA–60% (vol/vol) formamide, followed by RNA annealing by incubation for 3 h at 50°C. The hybridization reaction was precipitated with isopropanol, and the RNA was pelleted by centrifugation, resuspended in sample buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10% [vol/vol] glycerol, 0.1% Nonidet P-40, 0.01% bromophenol blue, and 0.01% xylene cyanol), and then electrophoresed on a 12% polyacrylamide gel in 45 mM Tris-borate–1 mM EDTA (pH 8.0). The separated duplex was localized by autoradiography, and the gel band was excised, pulverised, and extracted with 0.5 M ammonium acetate–0.1% SDS–10 mM EDTA (pH 8.0) for 2 h at room temperature. The eluted 3′RNA5 duplex was then extracted with phenol, precipitated with ethanol, and resuspended in a minimal volume of cold diethylpyrocarbonate-treated water and kept at −70°C until required.

To prepare the 3′RNA3 duplex the pEC-10B10-Hδ plasmid was initially used as a template for PCR amplification with primers which introduced a sequence complementary to the T7 promoter at the 3′ end of the positive-sense strand of the sequence (data not shown). The PCR product was digested at another internal *Sty*I site and used for in vitro transcription to produce an RNA complementary to the last 100 nucleotides of the coding strand of BTV S10 dsRNA. The transcript was recovered and annealed with the 3′RNA3 positive-sense release strand, and the derived duplex was purified as described above (Fig. 2, 3′RNA3).

To prepare a helicase substrate with a 5′ overhang (5′RNA duplex), a labeled 21-nucleotide-long release strand was constructed using the T7 promoter oligonucleotide and the protocols described above but with the template oligonucleotide 5′-AT GAT ACC ATT TCT CAA CCG CTA TAG TGA GTC GTA TTA CGC-3′ (the underlined sequence represents the sequence 21 nucleotides upstream of the 3′ end of the *Sty*I-cut plasmid pEC-10B10-Hδ). The labeled negative-sense product was recovered and annealed to the 128-nucleotide positive-sense T7 run-off transcript prepared from an *Sty*I-cut plasmid, and the derived 5′RNA duplex was purified as described above.

The blunt-end RNA duplex (Fig. 2, BluntRNA), corresponding to an internal fragment of BTV-10 S10 dsRNA 26 bp in length (underlined), was synthesized by preparing T7-produced labeled transcripts with the T7 promoter oligonucleotide and the following two synthetic oligonucleotides with corresponding T7 promoter sequences: 5′-T TTA GTG CGC GTG GAT GAT ACC ATT TTA TAG TGA GTC GTA TTA CGC-3′ and 5′-A AAT GGT ATC ATC CAC GCG CAC TAA ATA TAG TGA GTC GTA TTA CGC-3′. The products were recovered and annealed, and the derived duplex was purified as described above (Fig. 2, BluntRNA).

Helicase assay. To determine the optimal conditions for helicase activity the concentrations of $MgCl_2$ and ATP were initially tested in the concentration ranges of 1 to 10 mM and 1 to 15 mM, respectively, with standard reaction mixtures, which otherwise contained 30 mM Tris-HCl (pH 7.5), 10 mM DTT, and 1 μl of RNasin (Promega), as well as 5 fmol of labeled nucleic acid duplex substrate and 100 ng of purified VP6 protein. Subsequent reactions were performed with 3 mM $MgCl_2$ and 5 mM ATP, which gave the best results. Duplex unwinding was performed in 16-μl volume reaction mixtures incubated at 37°C

for 20 to 30 min and terminated by adding 4 μl of 5× SDS-PAGE sample buffer (100 mM Tris-HCl [pH 7.5], 50 mM EDTA, 50% [vol/vol] glycerol, 0.1% Triton X-100, 0.5% SDS, and 0.01% bromophenol blue). Samples were loaded onto 12% (wt/vol) or 18% (wt/vol) nondenaturing polyacrylamide gels in Tris-glycine buffer and electrophoresed at 100 V until the bromophenol blue dye approached the bottom of the gel. Gels were dried and exposed to X-ray film. Autoradiographs were aligned with the dried gels, the substrate and product bands were cut out, and the radioactivity associated with the gel pieces were quantitated with a liquid scintillation counter. From these data the percentages of substrate converted to released product were calculated.

Cross-linking of oxidized ATP with VP6. [α-^{32}P]-ATP (400 Ci/mmol) was oxidized with 0.5 mM sodium periodate and 0.5 mM HCl in the dark for 20 min. Excess periodate was consumed by the addition of glycerol, and the oxidized ATP (ox-ATP) was cross-linked with protein following the procedures described elsewhere (6). Briefly, 0.5 μg of protein was mixed with approximately 0.5 μM of ox-ATP in 50 mM Tris-HCl (pH 7.5)–5 mM $MgCl_2$–1 mM DTT, and the reaction was allowed to proceed overnight on ice in the presence of 7.5 mM $NaBH_3CN$. The incubation was stopped by the addition of an equal volume of 2× SDS-PAGE sample buffer, and protein complexes were loaded onto an SDS-10% (wt/vol) polyacrylamide gel to separate protein from the free nucleotides. After electrophoresis, the gels were dried and autoradiographed to determine the distribution of labeled protein.

ATPase assay. Except where indicated, ATPase assays involved incubating for 15 min at 37°C samples of 0.5 μg of VP6 in a 20-μl total volume of 30 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM DTT; 50 mg of bovine serum albumin per ml, 2 μg of poly(U), and 1 μCi of [α-^{32}P]ATP. After incubation, 2-μl samples were spotted onto polyethylenimine (PEI)-cellulose plates, and the chromatograms were developed in 0.75 M potassium phosphate. The plates were subsequently dried and autoradiographed. Control, nonradioactive nucleotide markers run alongside were identified using UV light.

A colorimetric assay for ATPase activity was carried out as described elsewhere (26). In short, a 3:1 mixture of 0.045% (wt/vol) malachite green hydrochloride and 4.2% (wt/vol) ammonium molibdate in 4 N H_2SO_4 was prepared, left for 20 min, and then filtrated. Samples of 50 μl of the ATPase reaction mixtures (containing 2 or 4 mM ATP) were combined after different time intervals with 800 μl of the malachite green hydrochloride-ammonium molibdate mix and left for 1 min at room temperature. Subsequently, 100 μl of 34% (wt/vol) sodium citrate in H_2O_2 was added to each reaction, the mixtures were incubated again for 30 min, and the colors that developed were read at 660 nm and compared to control reactions.

RESULTS

Does BTV VP6 possess helicase activity? A BTV helicase may be required to unwind the dsRNA genome of BTV prior to the initiation and during the synthesis of mRNA species (38). To determine whether VP6 possesses a helicase activity, the protein was expressed by using a recombinant baculovirus expression vector and purified to homogeneity as described in Materials and Methods (see Fig. 1). To assay the helicase activity of the protein, various RNA duplexes were generated as illustrated in schematic form in Fig. 2 and described in detail in the Materials and Methods.

Initial experiments for helicase activity of VP6 were undertaken with a partially duplex RNA corresponding to the 5′ end of BTV-10 S10 dsRNA and containing a 3′ overhang (Fig. 2, 3′RNA5). Standard reaction conditions with respect to the optimal levels of ATP and $MgCl_2$ were established (see Materials and Methods). As shown in Fig. 3A, VP6 liberated the labeled release strand from the partial duplex, and the activity was shown to be dependent on the addition of ATP and the presence of VP6 (Fig. 3B). The best results were obtained with a final concentration of 5 mM ATP. Higher concentrations (10 to 15 mM) produced an adverse effect (data not shown) as previously described for other helicases (55). As another negative control, purified BTV VP4 protein (30) was used in the helicase reactions in place of VP6. With VP4, no unwinding activity was detected (data not shown).

The effect of different pH conditions (pH 6.5 to 9.5) on the VP6 unwinding activity was also examined. The activity exhibited a broad pH requirement but was optimum in the region of pH 7.5 (Fig. 3C). When different concentrations of $MgCl_2$ were included in the reaction mixture, as shown in Fig. 3D, 3 mM $MgCl_2$ was found to be optimal. At 5 mM ATP, higher con-

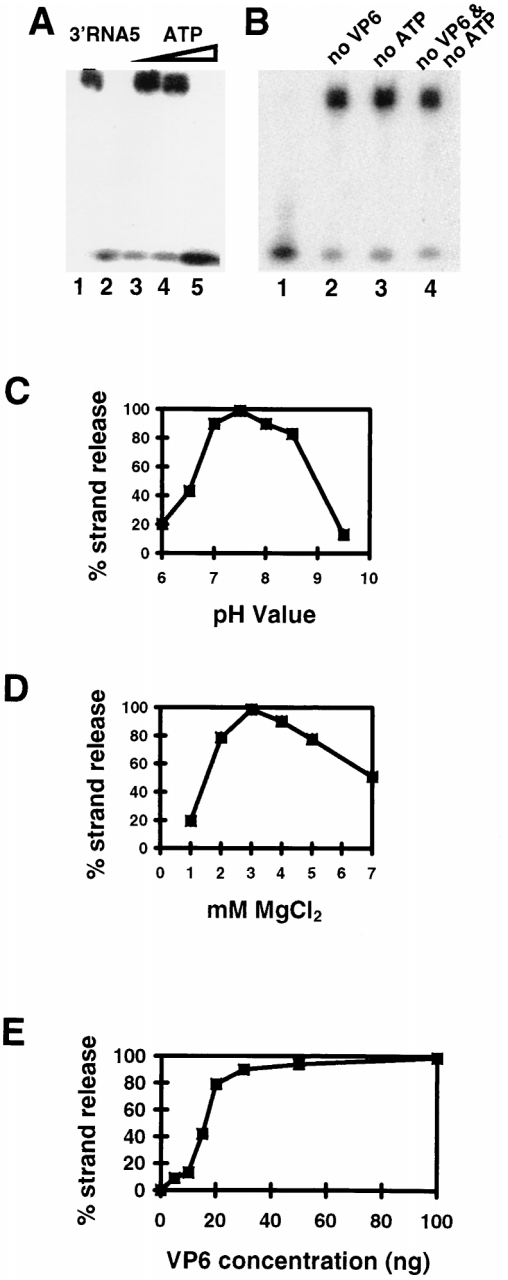

FIG. 3. RNA helicase activity associated with purified VP6 protein with (except as noted below) standard reaction conditions and substrates with 3′ overhangs. (A) Release of labeled RNA (lower bands) from duplexes (top bands) by using the substrate 3′RNA5 in the presence of increasing amounts of ATP. Lanes 1 and 2 represent reaction mixtures containing no added VP6 protein or boiled substrate only, respectively, and lanes 3 to 5 show the release of labeled RNA in the presence of 1, 2, or 5 mM ATP, respectively. (B) Release of labeled RNA from the duplex 3′RNA5. Lane 1, standard reaction mixture containing 5 mM ATP and VP6; lane 2, products of a reaction in which VP6 was omitted; lane 3, products of a reaction in which ATP was omitted; lane 4, products of a reaction where both ATP and VP6 were omitted. (C) Effects of buffers with different pH values on the percentage of labeled RNA released in otherwise standard reaction conditions with the 3′RNA5 substrate. The buffers used were HEPES-HCl for pH 6 to 7 and Tris-HCl for pH 7.5 to 9.5. (D) Effects of increasing concentrations of Mg^{2+} on the VP6-mediated helicase activity with the duplex 3′RNA5. (E) Effects of increasing VP6 protein concentrations on the RNA helicase activity.

centrations of $MgCl_2$ produced an adverse effect (Fig. 3D). $MnCl_2$ was also found to promote the unwinding reaction in lieu of $MgCl_2$, although to a lesser extent than $MgCl_2$ (data not shown). It is noteworthy that low levels of unwinding activity were also detected in the absence of added divalent cations but that this was abolished upon addition of 1 mM EDTA to the reaction (results not shown), suggesting that VP6 may contain some bound divalent cations. This issue was not examined further.

To determine the extent to which the helicase activity was dependent on the concentration of VP6 protein, various amounts (0 to 200 ng) of VP6 were used in standard reaction mixtures with constant amounts of substrate. As shown in Fig. 3E, under standard conditions the displacement of the labeled release strand was proportional in the lower concentration range to the amount of VP6 added to the reaction mixture.

The effect of salt on the RNA helicase activity was also tested. Concentrations that provided 5, 10, 20, 50, or 100 mM NaCl in otherwise standard reaction mixtures were assayed. At 5 and 10 mM NaCl, 50 and 30%, respectively, of the standard unwinding activity was observed (data not shown). Concentrations of 20 mM and higher proved to be totally inhibitory.

Since in other systems helicase activity is dependent on ATP hydrolysis, we examined the VP6 helicase activity in the presence of ribo- or deoxyribonucleoside triphosphates (Fig. 4). Only ATP efficiently promoted the unwinding of the 3′RNA5 substrate by VP6. The other triphosphates provided limited, if any, unwinding of the substrate (Fig. 4A).

Helicases are often only active on specific nucleic acid sequences. To determine whether the RNA unwinding activity of VP6 was also sequence specific, we prepared alternative substrates involving different sequences and overhangs. For partial duplexes with 3′ or 5′ overhangs (Fig. 2, 3′RNA3 or 5′RNA) the results obtained were similar to those obtained with the 3′RNA3 substrate (see Fig. 4B and C). These data suggest that the recognition of the substrate by the enzyme was not influenced by whether there was a 3′ or 5′ overhang. Since equally efficient unwinding activity of VP6 was observed with both types of substrate, the data suggest that VP6 has both 3′ to 5′ and 5′ to 3′ directionality.

VP6 can unwind blunt-end RNA duplexes. To address whether VP6 can unwind duplexes with blunt ends at both extremities, a duplex consisting of a 26-bp RNA was tested. Unwinding of this substrate was observed, albeit at a low (6%) level (Fig. 4D, lane 3). Little single-stranded product was observed in the absence of ATP (Fig. 4D, lane 1) or VP6 (Fig. 4D, lane 2). The data support the view that VP6 may be involved in the unwinding of blunt-ended substrates such as the viral dsRNA, although whether other factors are involved that enhance the efficiency of this activity was not investigated further.

BTV VP6 has an ATP binding domain. To determine whether VP6 binds ATP, an affinity labeling procedure was employed (6) to cross-link ATP to VP6. To accomplish this, ATP was initially oxidized (ox-ATP) before binding to VP6, and the complex was stabilized by subsequent reduction (see Materials and Methods). ox-ATP cross-linked efficiently to VP6, as shown in Fig. 5. When a similar quantity of bovine serum albumin (BSA) was used as a control, only a faintly labeled band was detected (Fig. 5). Such labeling has been considered nonspecific background labeling (37).

RNA-dependent ATPase activity of VP6 helicase. Similar to other helicases, and reflecting the requirement for ATP to unwind polynucleotide duplexes, the BTV VP6 protein is expected to possess an RNA-stimulated ATPase activity. This was demonstrated by the addition of a poly(U) homopolymer. Depending on the presence of VP6, as well as the presence of

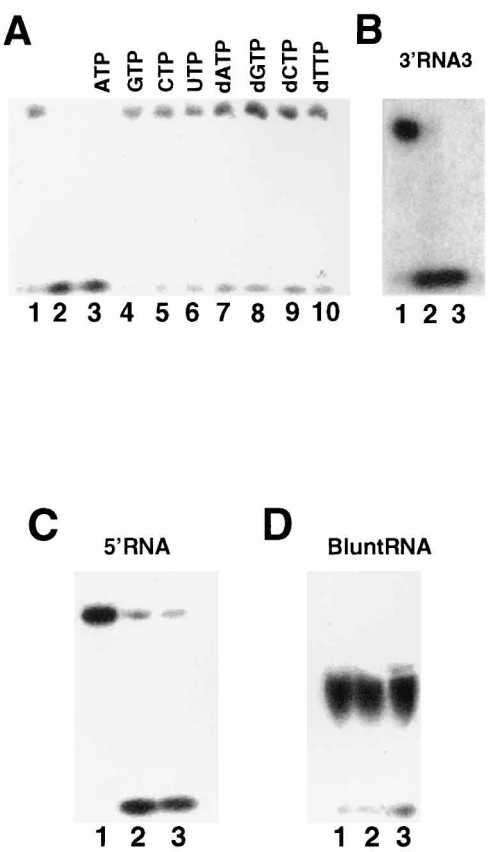

FIG. 4. Properties of BTV-10 VP6 helicase. (A) The effects of different ribonucleoside triphosphates were determined by using nucleotides at 5 mM in otherwise standard reaction conditions and substrate 3′RNA5. Lanes 1 and 2 represent reactions either without added VP6 protein or with a heat-denatured substrate, respectively. Lane 3 shows the products of a standard reaction with 5 mM ATP, and lanes 4 to 10 show the products of reactions with the indicated nucleoside or deoxynucleoside triphosphates in lieu of ATP. (B) Results of standard helicase incubations using the 3′RNA3 duplex substrate with controls either lacking protein (lane 1) or containing boiled substrate (lane 2) or in the presence of VP6 (lane 3). (C) Results of standard helicase incubations with controls either lacking protein (lane 1) or containing boiled substrate (lane 2) or with the 5′ RNA duplex substrate (lane 3). (D) Results of standard helicase incubations with controls lacking either ATP (lane 1) or VP6 (lane 2) or with the BluntRNA duplex substrate (lane 3).

polynucleotide, VP6 converted [α-^{32}P]ATP into labeled ADP, liberating free phosphate (Fig. 6A). Higher levels of hydrolysis were observed at levels of poly(U) of >0.05 μg per reaction. Amounts of 2 μg per standard reaction gave significant levels of ADP (Fig. 6B). The lack of nucleic acid specificity for the ATP hydrolysis reaction was demonstrated through the use of the alternative RNA homopolymers poly(A), poly(C), and poly(G) (Fig. 6B). The apparent differences in the extents of the hydrolyses may be due to the different structures of the homopolymers; however, this issue was not investigated further. The lack of requirement for specific sequences matches the previously described nonspecific RNA-binding of VP6 (41). This observation is further supported by the unwinding activity obtained for substrates with different 3′ overhangs (Fig. 3). Additionally, ATP hydrolysis was stimulated by the double-stranded homopolymer poly(A) · poly(U) (Fig. 6C). ATP hydrolysis was not affected when DTT (Fig. 7A, lane 2) or BSA (Fig. 7A, lane 3) was omitted, nor was it affected when either 3 mM $MgCl_2$ (Fig. 7A, lane 4) or 1 mM $MnCl_2$ (Fig. 7A,

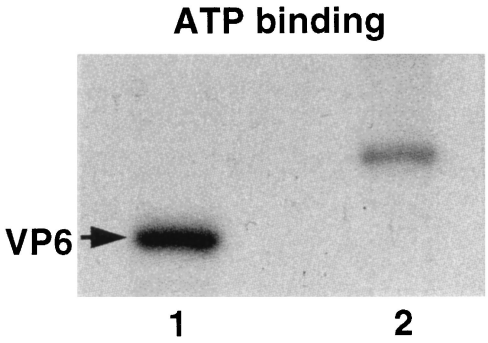

FIG. 5. SDS-PAGE autoradiography analysis of cross-linking oxidized [α-^{32}P]ATP (ox-ATP) to BTV-10 VP6. Lane 1 shows BTV-10 VP6 cross-linked with ox-ATP, and lane 2 shows a control cross-linking of ox-ATP with BSA.

lane 5) was omitted from the reaction mixtures, although only a trace of ATPase activity was observed when neither Mg^{2+} nor Mn^{2+} was present (Fig. 7A, lane 6). However, this residual activity was abolished by the addition of 1 mM EDTA (Fig. 7A, lane 7). The hydrolysis of ATP by VP6 was shown to be time dependent, as shown in Fig. 7B with two different substrate concentrations.

DISCUSSION

Experimental evidence of RNA helicase activity has been reported for a few viral proteins, but no such activity has so far been demonstrated to be associated with a particular protein of any dsRNA virus. In this study, a procedure for obtaining large quantities of purified, enzymatically active BTV-10 VP6 protein is reported. The purified VP6 possesses a number of properties associated with its helicase function. It binds ATP, exhibits an RNA-dependent ATPase activity that requires the presence of divalent cations and which involves the removal of the terminal phosphate, and in vitro unwinds various forms of RNA duplexes in the presence of ATP. Other nucleoside triphosphates were much less efficient in promoting the RNA unwinding reaction. Both ss- and dsRNA stimulate the ATPase activity.

With respect to directionality, BTV-10 VP6 helicase unwound substrates with 3′ overhangs similar to the helicases described to date that exhibit a 3′ to 5′ directionality (2, 8, 24, 47, 52). In addition, VP6 unwound substrates with a 5′ overhang. This type of activity has been described in only a few cases, such as with the bacterial termination factor rho (3) and the eukaryotic translation initiation factors 4A and 4F (42), where 5′ to 3′ unwinding is stimulated by the presence of RNA 5′ cap structures. Among viral helicases, the bidirectional form of unwinding has been described for the simian virus 40 large T-antigen DNA helicase (31, 54) and where the hexameric T-antigen complex acts unidirectionally, but upon forming a binary hexameric complex, DNA is unwound bidirectionally. Helicases have been shown to be involved in some steps of transcription (4), and in this context the feature of bidirectional unwinding might enable the BTV VP6 to function in different steps of the virus replication process in addition to helicase functions associated with mRNA formation.

In general, helicases have been shown to require single-stranded regions for initial attachment (10), although it has been suggested that RNA helicases should recognize dsRNA (28). VP6 binds dsRNA nonspecifically (41); however, using the BluntRNA substrate we have shown that some unwinding is obtained with this substrate. Whether the presence of a cap

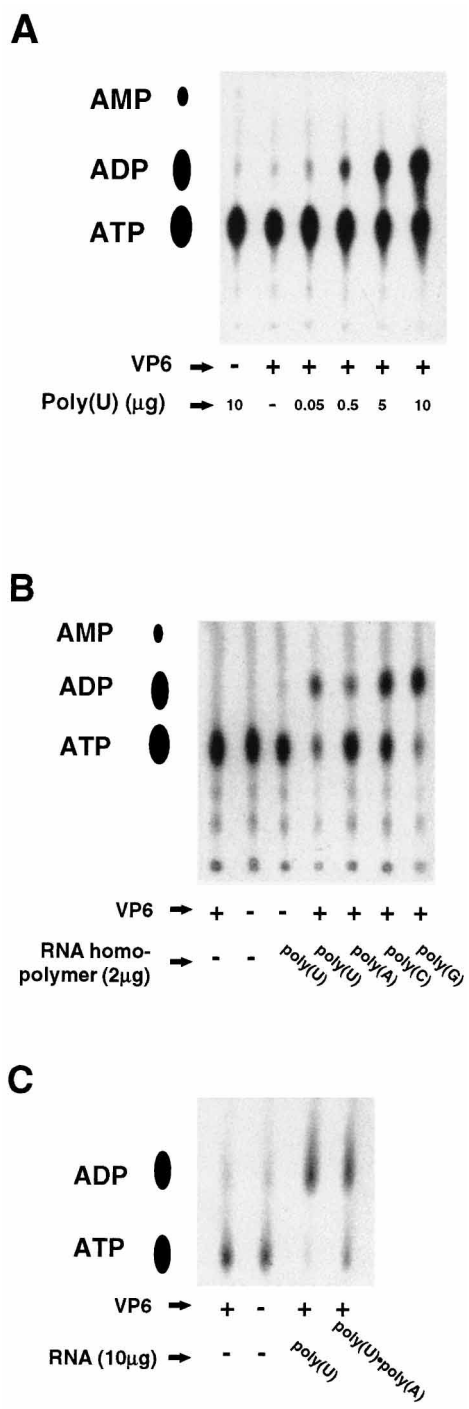

FIG. 6. Autoradiography of PEI-cellulose plates demonstrating the RNA-stimulated ATPase activity of BTV-10 VP6. (A) The hydrolysis of ATP by VP6 is shown as a function of the indicated amounts of poly(U) included in the reaction mixture. Two controls are shown on the left, one in which VP6 was omitted and the other in which poly(U) was omitted. The positions of the reference AMP, ADP, and ATP are shown. (B) The effects of the indicated RNA homopolymers (at 2 μg per reaction) on the ATPase activity of VP6 are shown. Controls, shown on the left, included the addition of no RNA homopolymer, no RNA homopolymer and no VP6, and no VP6. The positions of the reference AMP, ADP, and ATP are shown. (C) The effects of poly(U) (ssRNA) and poly(A) · poly(U) (dsRNA) on the ATPase activity of VP6 are shown. Controls are shown on the left, one in which RNA was omitted and the other in which both RNA and VP6 were omitted. The positions of the reference ADP and ATP are shown.

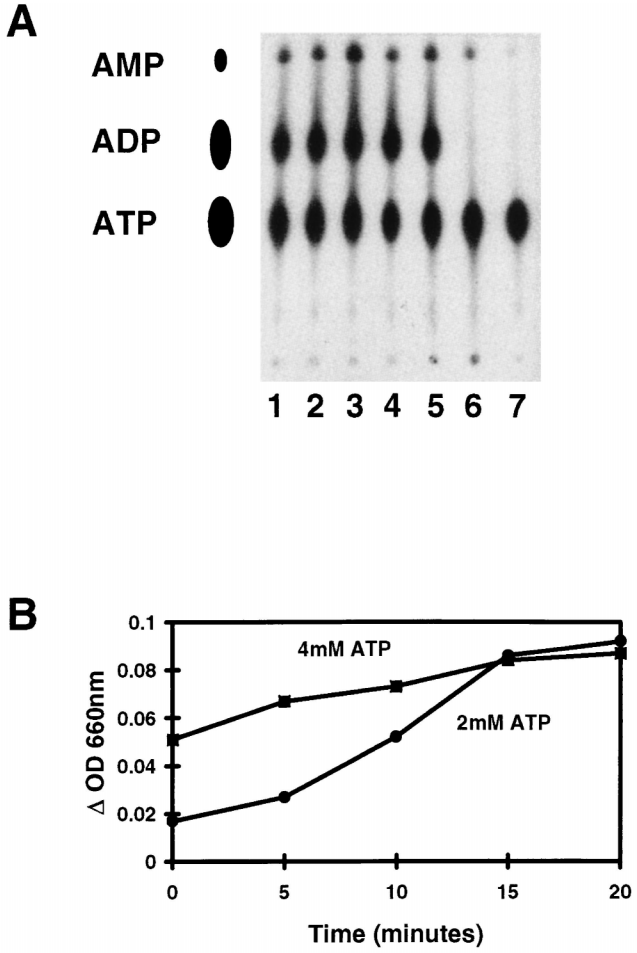

FIG. 7. ATPase activity of BTV-10 VP6. (A) Autoradiograph of a PEI-cellulose plate demonstrating the RNA-stimulated ATPase activity of BTV-10 VP6 in standard reaction mixtures (lane 1) or in reaction mixtures lacking DTT (lane 2), BSA (lane 3), Mg^{2+} (lane 4), Mn^{2+} (lane 5), both Mg^{2+} and Mn^{2+} (lane 6), or both Mg^{2+} and Mn^{2+} but in the presence of 1 mM EDTA (lane 7). (B) Time course of ATP hydrolysis analyzed by the colorimetric measurement of released inorganic phosphate (31) at the indicated ATP concentrations. The optical density (OD) at 660 nm represents the OD of the samples after subtracting the OD of control samples lacking VP6.

structure at one end of the duplex would enhance unwinding of an RNA substrate with blunt ends has not been determined. The initiation of unwinding at blunt ends has previously been described for cellular helicases such as the *Escherichia coli* DNA helicase II (43) but has not been shown to date for any viral helicase. In view of the possibility that the BTV VP6 may unwind the blunt ends of an RNA, no definitive conclusion can be drawn about the directionality of the unwinding catalyzed by BTV VP6.

It is not yet clear whether all of the nucleic acid binding sites of VP6 are necessary for the binding of dsRNA during the helicase reaction. The data obtained for the RNA helicase A from *Drosophila melanogaster* and *Caenorhabditis elegans* suggest that the enzymatic domain possesses a single RNA-binding site, which also promotes ATPase activity (11), although the protein contains additional distinct RNA-binding domains. RNA-binding domains located around positively charged arginine-rich motifs (46) and RGG repeats have been noted previously (25). Clusters of RGG repeats have been proposed as an RNA-binding motif (20), as well as functioning as nuclear localization signals (32). Interestingly, with appropriate deletion mutations VP6 has been shown to possess nuclear localization signals (56), and one putative RNA-binding region has been identified in VP6 which contains an RGG site within an arginine-rich stretch. Whether this area constitutes the main RNA-binding site has yet to be elucidated. Further, the same area has an amino acid sequence similar to the conserved motif of DNA and RNA helicases of the superfamily II (22), which have been demonstrated to be involved in RNA binding and ATPase activities (36). This conserved motif is represented by the sequence QRxGRxxR. In VP6 the Q is replaced by E (ERGGRKQR). Despite this substitution, this region exhibits an overall positive charge, consistent with a role in ATP hydrolysis. Moreover, this pattern is conserved in the VP6 proteins of other BTV serotypes, such as BTV-11, BTV-13, and BTV-17 (16).

Nucleic acid-binding regions of the BTV VP6 might have other functions, possibly allowing VP6 to participate in virus assembly by interacting with the 10 mRNA species and thereby assisting in the process of synthesizing core particles containing a precise set of mRNA and subsequently dsRNA segments. It is also possible that there is a switching between these functions, perhaps through interactions with other components of the viral particle or with cellular components like the cytoskeleton (18) during early stages of morphogenesis. In this respect it has also been proposed that there are different forms of VP6 within the BTV-1 particle (51), resulting from separate initiation of translation at distinct start codons. Similar results with two other orbiviruses, epizootic hemorrhagic disease virus (33) and African horsesickness virus (14), as well as with VP7 of simian rotavirus SA11 (5), suggest that these different forms might exhibit distinct functions.

By using mutational analysis of the above-mentioned motif of helicase superfamily II, it has been suggested that the RNA binding, ATP hydrolysis, and helicase activities are coupled (36). It remains to be determined if the related motif in VP6 performs these functions or whether ATP binding is mediated by another region of VP6, exhibiting similarities with the purine nucleoside triphosphate-binding motif A (P loop) described in other systems (12, 44). Two areas in the VP6 sequence show partial homology to this motif (G/AxxGxGKS/T). Both regions differ by one residue from the motif A sequence, having either the sequence ANRGDGKV for one region or ADAGVGAT for the other region. Nevertheless, they might account for the ATP binding activity of VP6, as similar modifications to this motif have been found in other ATP binding proteins (23).

Apart from the similarities with previously recognized motifs, computer-assisted analysis using the GCG version 8 set of programs (Genetics Computer Group, Madison, Wis.) has revealed no clear structural analogies of VP6 with the putative helicase proteins of other members of the *Reoviridae* family or with other published helicases. Possible functional analogies between helicases of different organisms have been discussed (53); however, such discussions have generally excluded viral proteins due to lack of experimental evidence for their proposed function. However, the BTV VP6 protein performs functions analogous to those of certain prokaryotic and eukaryotic helicases which are not observed for other viral proteins. In view of this it might therefore represent a new class of viral helicases not previously identified to date. Further structural and functional characterization of BTV-10 VP6 will help to unveil its particular features and establish its key role in BTV transcription and in other aspects of BTV replication and morphogenetic functions.

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