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Purified Recombinant Bluetongue Virus VP1 Exhibits RNA Replicase Activity

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The polymerase protein of all known double-stranded RNA (dsRNA) viruses is located within a complex subviral core particle that is responsible for transcription of the viral genome. For members of the family *Reoviridae*, this particle allows messenger sense RNA synthesis while sequestering the viral genome away from cellular dsRNA surveillance systems during infection of eukaryotic cells. The core particle of bluetongue virus (BTV) consists of the major structural proteins VP3 and VP7 and the minor enzymatic proteins VP1 (polymerase), VP4 (capping enzyme), and VP6 (helicase). In this report we have characterized fully processive dsRNA synthesis by VP1 from a viral plus-strand RNA template in the absence of the other proteins of the BTV core. This replicase activity consists of de novo initiation of synthesis, followed by elongation of the minus strand. Purified VP1 exhibits little sequence specificity for BTV plus-strand template, suggesting that the choice of viral over nonviral RNA template comes from its association with other proteins within the viral core.

The double-stranded RNA (dsRNA) genome of members of the family *Reoviridae* remains closely associated to a multilayered icosahedral inner capsid following partial uncoating of the virion during cell penetration. This inner capsid forms a transcriptionally active core particle (1, 5, 26) that acts to protect the viral genome from cellular dsRNA surveillance systems. Since the accessibility of cellular dsRNA responsive proteins to the viral genome must be tightly controlled, it is necessary for the core to contain all of the enzymatic activities required to produce capped viral plus-sense RNA. For members of the *Reoviridae*, it has been demonstrated that rotavirus cores (and core-like particles) that have been treated to remove the viral genome and incubated with viral plus-strand RNA are capable of dsRNA synthesis (4). Furthermore, this dsRNA synthesis activity is highly specific to plus-strand RNA that contain authentic viral sequences at the 3′ termini (2, 3, 35). Recent studies have revealed that although the purified rotavirus polymerase protein (VP1) is capable of binding specifically to viral plus-strand RNA it is not capable of initiating dsRNA synthesis in the absence of the subcore protein (VP2) (20, 31, 36). In contrast, the structure of the reovirus polymerase (λ3) has been determined by X-ray crystallography, and it has been demonstrated that these crystals are capable of nonspecific initiation and limited elongation of dsRNA synthesis (30). The nonspecific initiation activity associated with λ3, which allows it to initiate dsRNA production on a nonviral template (5′-UA GCCCCC-3′), is much more similar to the activity described for the polymerase protein (P2) of the dsRNA bacteriophage ϕ6 (15). P2 does have a preference for templates with the authentic phage-RNA-like dinucleotide at the 3′ end of the template RNA (13) but it will catalyze dsRNA synthesis on any template single-stranded RNA (ssRNA) (14, 15).

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Bluetongue virus (BTV) belongs to the *Orbivirus* genus of the *Reoviridae*. The virion is a nonenveloped, triple-layered, icosahedral particle containing 10 linear dsRNA genome segments. Like other members of the *Reoviridae*, the outer capsid of the viral particle, consisting of the VP2 and VP5 proteins, is lost after entry into a host cell. The remaining transcriptionally active icosahedral core particle consists of the major proteins VP3 and VP7, the enzymatic minor proteins VP1, VP4, and VP6, and the dsRNA genome (24). A high-resolution atomic structure of the core is available (8). This structure reveals that the viral dsRNA is enclosed by a subcore shell composed of 120 copies of VP3, which is itself surrounded by a layer of 260 trimers of VP7. Densities consistent with the presence of the minor core proteins were located at the fivefold axes of the core particle (8). This has been confirmed by recent cryoelectron microscopy reconstruction analyses (B. V. V. Prasad, unpublished observations). We have previously expressed all three of the minor core proteins of BTV in insect cells by baculovirus expression systems and have demonstrated that purified VP4 and VP6 have the specific activities of capping and helicase enzymes, respectively (6, 11, 22, 23, 29). We have also shown that infected clarified insect cell extracts containing soluble recombinant VP1 can extend an oligo(A) primer in the presence of a poly(U) template, indicating that it is the viral polymerase protein (32).

We demonstrate here that purified VP1, in the absence of all other virus-encoded proteins and structures, can act as the BTV replicase protein synthesizing dsRNA from a viral plus-strand RNA template. In addition, we show that, like the activity of the reovirus λ3 and bacteriophage ϕ6 P2 but unlike the rotavirus "open core" system, the replicase activity of BTV VP1 is largely template independent. To our knowledge, this is the first demonstration that the purified polymerase protein of a member of the *Reoviridae* is capable of fully processive synthesis of a complete dsRNA genome segment from a plus-strand template RNA in the absence of other viral proteins.

MATERIALS AND METHODS

Viruses and cells. *Spodoptera frugiperda* (Sf9) cells were grown in Sf900-II medium (Gibco) by using standard techniques (9). Recombinant *Autographica californica* nuclear polyhedrosis virus (AcNPV) expressing N-terminally His-tagged VP1 of BTV serotype 10 (AcBTV10.NHis1) was expressed in Sf9 cells (see below).

Construction of a recombinant baculovirus expressing VP1. The baculovirus transfer vector pBluebacHisAVP1 was constructed as follows: a DNA fragment containing the coding region of BTV 10 segment L1 flanked by a BamHI site and a PstI site was ligated to pBluebacHisA (Invitrogen), which had been digested with BamHI and PstI. Recombinant baculovirus expressing VP1 (AcBTV10.NHis1) was made by cotransfecting pBluebacHisAVP1 with Bac-N-Blue linear *A. californica* nuclear polyhedrosis virus DNA (Invitrogen) into Sf9 cells. The recombinant baculovirus was selected and plaque purified on the basis of the ability to form blue plaques in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and to express a protein of the expected size.

Expression and purification of recombinant VP1. VP1 was routinely expressed in Sf9 cells; cells were grown to a density of 2×10^6 cells/ml in Sf900-II medium and infected at a multiplicity of infection of 2.5 with AcBTV10.NHis1. Cells were harvested at 64 h postinfection and washed once in phosphate-buffered saline before being stored at −70°C until required.

Cell pellets were thawed and resuspended at a cell density of 2×10^7 cells/ml in 50 mM NaH_2PO_4 (pH 8.0)–10% glycerol. NP-40 was added to a final concentration of 0.5%, and the cells were stirred at 4°C for 10 min to lyse them. The lysate was then centrifuged at 3,500 × *g* for 10 min in a bench-top centrifuge to remove debris, and the supernatant was decanted and stored on ice. The lysis and centrifugation steps were repeated on the pellet. One milliliter of Ni-NTA resin (Qiagen) was preequilibrated in 50 mM NaH_2PO_4 (pH 8.0)–10% glycerol, and the two supernatants were combined and batch bound to the resin for 1 h at 4°C. The resin was poured into an empty 15-ml column and washed with 200 ml of wash buffer (50 mM NaH_2PO_4 [pH 8.0], 10% glycerol, 10 mM imidazole), and VP1 was eluted in the same buffer containing 250 mM imidazole. Fractions containing VP1 were combined and diluted to 50 mM imidazole in 50 mM Tris-HCl (pH 7.5)–1 mM dithiothreitol (DTT)–10% glycerol. A 1-ml heparin column (Amersham Biosciences) was equilibrated in the same buffer by using an AKTA purifier system (Amersham Biosciences), and the eluate from the Ni-NTA resin was loaded. The column was washed with 10 column volumes of the same buffer, and bound proteins were eluted with a linear NaCl gradient over 20 column volumes. VP1 eluted at 350 to 375 mM NaCl and was the major peak. The purity of recombinant VP1 was >95% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie blue R250. The protein concentration was estimated by using the Bradford assay. Purified VP1 was dialyzed against two changes of Tris-HCl (pH 7.5)–10% glycerol–1 mM DTT, and VP1 was stored in aliquots at −20°C.

Immunoblotting. Standard immunoblotting techniques were used with a rabbit polyclonal antiserum raised against BTV; samples were visualized by using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma).

Preparation of RNA substrates for use in the replicase assay. Synthetic ssRNAs were prepared by runoff in vitro transcription with T7 RNA polymerase. Templates for in vitro transcription were prepared by digestion of plasmid DNA with the appropriate restriction endonuclease. The digested plasmids were successively extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and an equal volume of chloroform and then precipitated with an equal volume of isopropanol. The DNA was pelleted by centrifugation at 21,000 × *g* and washed twice with 200 μl of 70% (vol/vol) ethanol. DNA pellets were dried at 37°C and dissolved in 10 mM Tris-HCl (pH 8.0). Agarose gel electrophoresis was performed to confirm that digests were complete. The digested plasmid DNAs were used as templates with the T7 RiboMax large-scale RNA production system (Promega) according to the manufacturer's instructions. Briefly, restriction endonuclease digested plasmid DNA was included at 100 ng/μl in reactions containing 7.5 mM ATP, 7.5 mM GTP, 7.5 mM UTP, 7.5 mM CTP, 80 mM HEPES-KOH (pH 7.5), 24 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, and T7 RNA polymerase. Labeled ssRNAs were prepared as described for unlabeled RNAs, except that the CTP concentration was reduced to 750 μM and [α-^{32}P] CTP (3,000 Ci/mmol; Amersham Biosciences) was included at 0.25 μCi/μl. Reactions were incubated at 37°C for 3 h and stopped by the addition of 1 U of RQ1 RNase-free DNase (Promega) per μg of DNA template. Incubation was continued at 37°C for 30 min. Reaction mixtures were successively extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and an equal volume of chloroform and then purified through a Sephadex G-25 MicroSpin column (Amersham Biosciences). RNA preparations were precipitated with an equal volume of isopropanol, resuspended in diethyl pyrocarbonate-treated distilled water, and stored at −80°C. The RNA concentrations were determined from the absorbance at 260 nm, and the integrity of the RNA was determined by electrophoresis prior to use in the replicase assay.

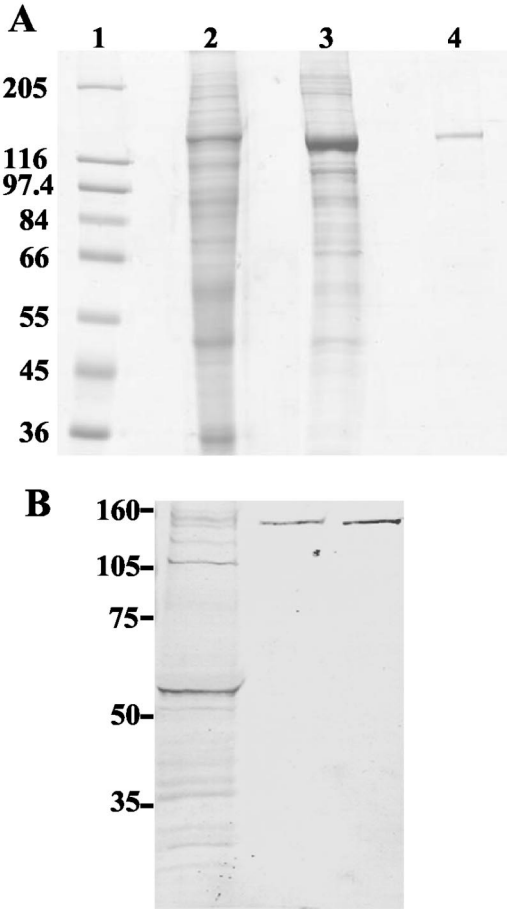

FIG. 1. Purification of His-tagged BTV 10 VP1 from recombinant baculovirus-infected Sf9 cells. (A) Stages in the purification of His-tagged VP1. Protein samples were resolved by SDS–10% PAGE prior to staining with Coomassie blue R250. Lane 1, protein molecular mass markers (Sigma), indicated in kilodaltons; lane 2, lysate of AcBTV10.NHis1-infected Sf9 cells; lane 3, protein eluted from Ni-NTA resin; lane 4, protein eluted from heparin column. (B) Immunoblotting of purified His-tagged VP1, with a BTV-specific antiserum. Lane 1, lysate of AcBTV10.NHis1-infected Sf9 cells; lane 2, VP1 after Ni-NTA purification; lane 3, VP1 after heparin column purification. Sizes of prestained protein molecular mass markers are indicated in kilodaltons.

Denaturing agarose gel electrophoresis. T7 RNA polymerase in vitro-synthesized RNAs or products of the replicase assay (see below) were analyzed by electrophoresis on 1% agarose in morpholinepropanesulfonic acid (MOPS) electrophoresis buffer in the presence of formaldehyde by using standard techniques (25). RNA markers (Promega) were used as ssRNA size markers. For phosphorimager analysis, gels were dried between cellophane sheets and exposed to phosphor screens. Images were obtained by using a Molecular Dynamics Storm 840 PhosphorImager, and quantitation was performed by using ImageQuant 5.0 software.

Optimized replicase assay. In vitro-synthesized RNAs were denatured prior to use in the replicase assay, as follows. RNA was made up to 6.25 μl in diethyl pyrocarbonate-treated water; then 2.5 μl of 0.1 M methylmercury(II) hydroxide was added, followed by incubation at room temperature for 10 min. Next, 2.5 μl of 10% 2-mercaptoethanol was added, and the mixture was incubated at room temperature for 5 min. To assay the replicase activity of VP1, the denatured RNA was immediately added to the remaining reaction components to give the following reaction conditions in a 50-μl volume: 50 mM Tris-HCl (pH 7.5); 160 μM (each) ATP, GTP, and UTP; 4 μM CTP; 6 mM magnesium acetate; 1 mM DTT; 4 μg of actinomycin D/ml; 2% (wt/vol) polyethylene glycol 4000; 2 U

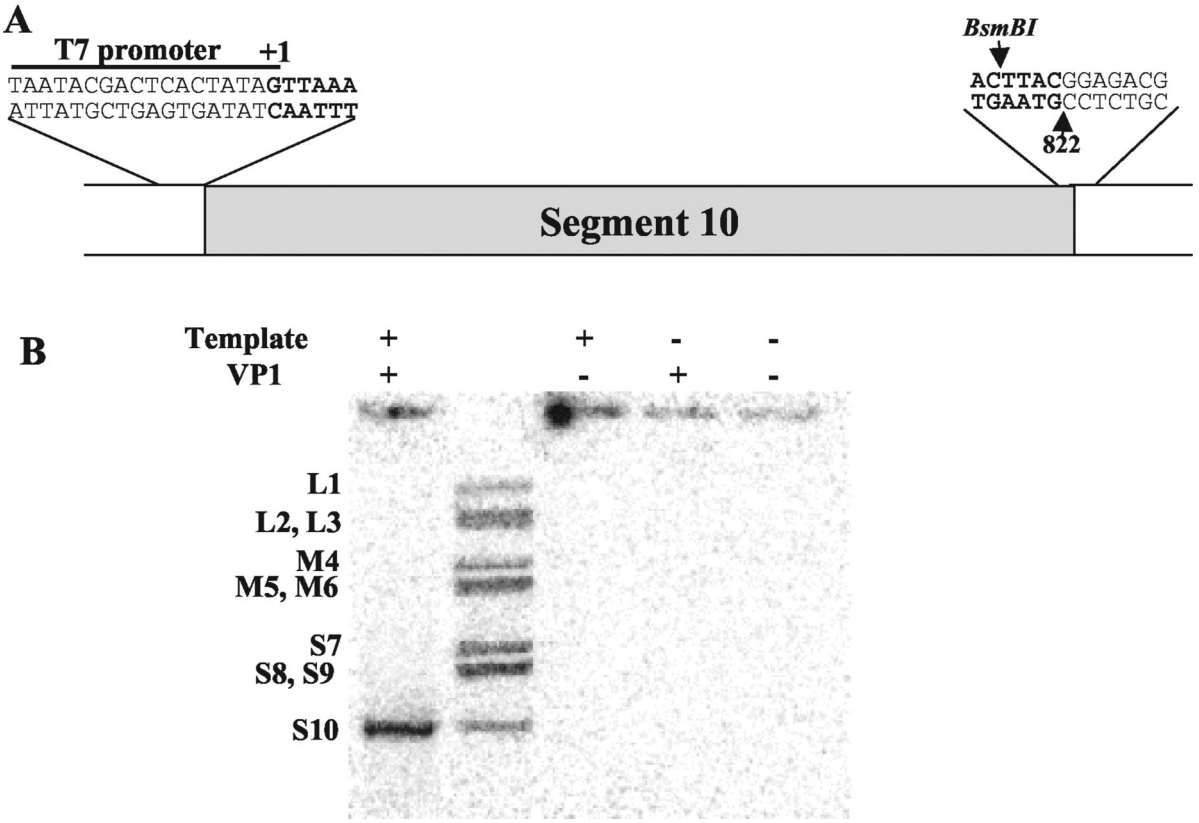

FIG. 2. In vitro replicase activity of purified recombinant VP1. Template RNA for the replicase assay was produced by in vitro transcription of the construct shown in panel A after linearization with BsmBI. BTV sequences are indicated in boldface. Restriction endonuclease cleavage sites are indicated with arrows. (B) Autoradiograph of products from the in vitro replicase reaction with S10 plus-strand RNA template. Inclusion or omission of reactants in the replicase assay is indicated by "+" and "−," respectively. End-labeled BTV genomic dsRNA is included in the second lane from the left, and the migration of segments L1 to S10 is indicated. Reactions were performed with 2.5 μg of VP1 and 2.5 μg of S10 plus-strand template RNA at 37°C for 4 h. Products were resolved by 9% PAGE.

of RNasin (Promega)/μl; 0.1 μCi of [α-^{32}P]CTP (3,000 Ci/mmol; Amersham Biosciences)/μl, 50 ng of VP1/μl, and 50 to 100 ng of template RNA/μl. In control reactions, VP1 was replaced with VP1 storage buffer (50 mM Tris-HCl [pH 7.5], 1 mM DTT, 10% glycerol), and denatured RNA was replaced with a denaturation mix lacking RNA. Reactions were incubated at 37°C for 4 h and stopped by extraction with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). Products were precipitated with an equal volume of isopropanol in the presence of 10 μg of wheat germ tRNA (Sigma) as a carrier.

Purification and labeling of BTV dsRNA. BTV dsRNA was purified from BTV 10-infected BSR cells as described by Mertens et al. (16). Complete BTV dsRNA was labeled by the method of England and Uhlenbeck (7). Briefly, 5 to 10 μg of BTV dsRNA was 3′ end labeled in a 30-μl reaction in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 10% dimethyl sulfoxide, 0.3 μCi of cytidine-3′,5′-bis[α-^{32}P]phosphate/μl, and 0.67 U of T4 RNA ligase/μl at 4°C for 16 h. The reaction was stopped by extraction with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and the products were precipitated with an equal volume of isopropanol. The products were dissolved in 20 μl of Tris-EDTA (pH 8.0) and stored at −20°C.

Nondenaturing PAGE. Products of the replicase assay were resolved on 9% polyacrylamide gels (PAGE) run in Tris-glycine buffer as follows. Replicase assay products were dissolved in 7 μl of loading buffer (Tris-glycine buffer, 50% glycerol, 0.4% bromophenol blue) and electrophoresed on 9% PAGE gels in Tris-glycine buffer. 3′-End-labeled BTV 10 dsRNA was used to provide dsRNA size markers. Gels were dried and exposed to phosphor screens, and images were quantitated as described above.

RESULTS

Expression and purification of His-tagged recombinant VP1 protein. Our previous report of oligo(A) extension on a poly(U) template was performed with the recombinant baculovirus-infected, clarified, insect cell lysate (32). To investigate the specificity of polymerase activity of BTV VP1 in the absence of other viral proteins, an amino-terminal His-tagged VP1 was generated and expressed in baculovirus-infected Sf9 cells as described in Materials and Methods. A 150-kDa protein corresponding to the predicted size for VP1 was visualized by SDS-PAGE and Coomassie blue staining of AcBTV10.NHis1-infected Sf9 lysates (Fig. 1A, lane 2). The soluble protein in the lysate was purified by using nickel affinity chromatography (Fig. 1A, lane 3) and further purified by cation-exchange chromatography with heparin as the ligand (Fig. 1A, lane 4) as described in Materials and Methods. Typically, 0.8 mg of purified VP1 was obtained per liter of AcBTV10.NHis1-infected Sf9 culture. The identity of the 150-kDa protein was confirmed by immunoblotting with a BTV-specific antiserum (Fig. 1B). A C-terminally His-tagged variant of VP1 was also produced in the baculovirus system, but it was found that a greater proportion of the recombinant protein was present in the insoluble fraction than in the N-terminally tagged variant (data not shown). As a result the N-terminally tagged VP1 recombinant was used in all subsequent replicase assays.

Recombinant VP1 exhibits replicase activity. Having established that recombinant VP1 could be substantially purified

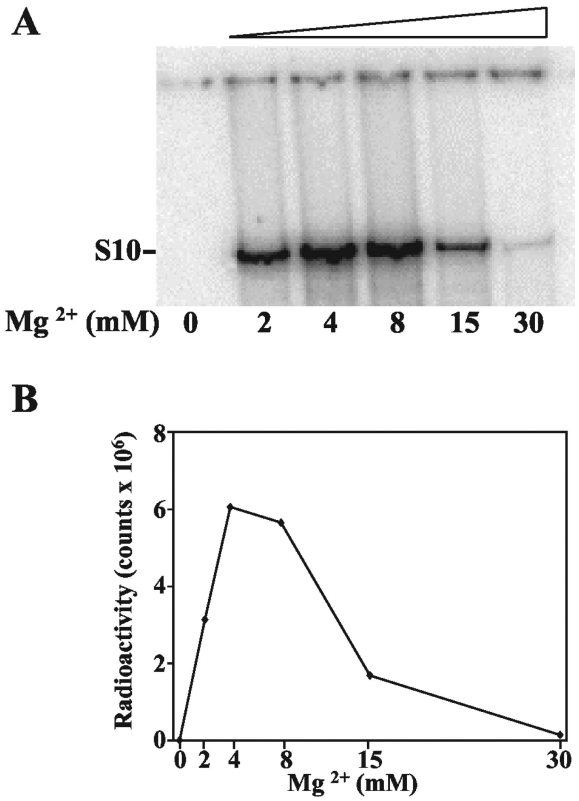

FIG. 3. Effect of Mg^{2+} concentration on replicase activity. Replicase assays were performed with 2 μg of purified VP1 and 2 μg of S10 plus-strand template RNA and various concentrations of magnesium acetate, as indicated. (A) Autoradiograph showing migration of labeled dsRNA products. (B) Graph showing relative amounts of radiolabeled dsRNA from replicase reaction as determined by phosphorimager analysis.

from cellular proteins, we have subsequently utilized this purified protein to examine its enzyme activity. Purified VP1 was tested for its ability to synthesize the cRNA strand from a viral plus-strand RNA template. Apart from the purified VP1 protein, the synthesis of dsRNA was assayed in the presence of Mg^{2+}, ribonucleoside triphosphates, and [α-^{32}P]CTP by using the BTV plus-strand transcript of segment S10. The S10 plus strand was synthesized by in vitro transcription with a BsmBI-digested plasmid, pS10BsmBI (Fig. 2A). This plasmid was designed such that linearization with BsmBI in this way produced a template that would result in the transcription of RNA that had a 3′ end that was identical to authentic viral S10 plus-strand RNA. After incubation of VP1 with S10 plus-strand template under these conditions, a radiolabeled RNA was produced that comigrated with labeled BTV genomic dsRNA when resolved by PAGE (Fig. 2B). The synthesis of S10 dsRNA in this assay was completely dependent on the presence of VP1 and template RNA; omission of either of these components of the reaction mix resulted in no detectable replicase activity (Fig. 2B). The polymerase activity of VP1 had an optimal Mg^{2+} concentration of between 4 and 8 mM (Fig. 3). This is consistent with the optimal concentration required for transcription from intact, authentic viral cores isolated from infected cells (33, 34). A time course of replicase activity for recombinant VP1 with 6 mM magnesium acetate, 1 mM DTT, 4 μg of actinomycin D/ml, and 2% (wt/vol) PEG 4000 in 50 mM Tris-HCl (pH 7.5) revealed that detectable replicase activity continued for at least 23 h and that the rate of the reaction was essentially constant for the first 4 h (Fig. 4). This prolonged activity is consistent with a low rate of depletion of the pool of template RNA molecules. The titration of PEG 4000, DTT, and actinomycin D had little effect on the replicase assay (data not shown). These optimized assay conditions were therefore used in further investigations.

The replicase activity associated with VP1 was not limited to a template derived from the plus-strand S10 segment of genomic dsRNA. At 822 nucleotides (nt), S10 is the smallest of the BTV genome segments. In order to test whether the synthesis of dsRNA from S10 template was representative of other BTV plus-strand RNA templates, we prepared transcripts of the largest BTV genome segments, L1 (3,954 nt) and L2 (2,926 nt), as well as the medium-size segment M4 (2,011 nt). Using equivalent constructs to those described for S10, we were able to successfully produce plus-strand in vitro transcripts for all three of these genome segments. When included in the replicase assay, these transcripts resulted in the production of dsRNA of a size equivalent to the authentic BTV genomic RNA (Fig. 5, lanes 2 to 5).

In order to confirm that the RNA product of the replicase

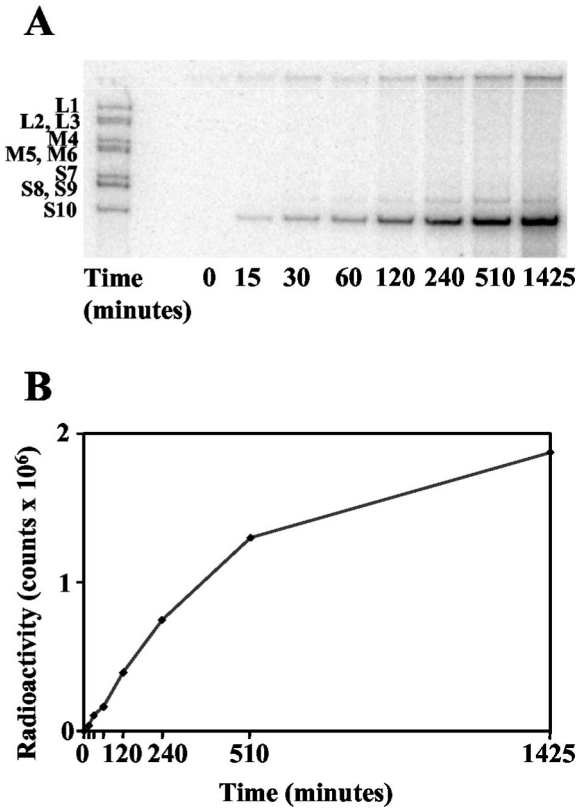

FIG. 4. Time course of VP1 replicase activity. The replicase assay was performed with 1 μg of purified VP1 and 1 μg of S10 plus-strand RNA for the times indicated. The reaction was stopped by the addition of an equal volume of phenol-chloroform. (A) Reaction products were resolved by nondenaturing 9% PAGE. (B) Graph showing phosphorimager analysis of radioactivity in dsRNA replicase product. The migration of end-labeled BTV genomic dsRNA segments L1 to S10 is indicated.

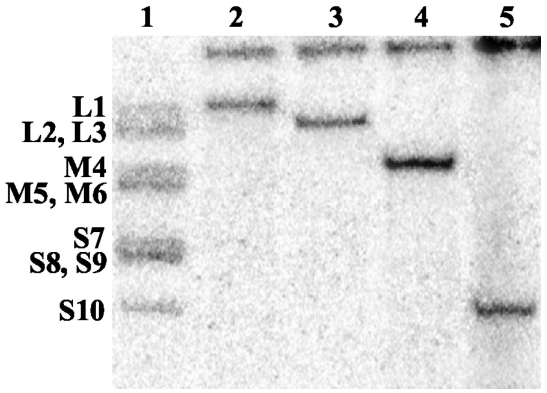

FIG. 5. VP1 replicase activity with diverse BTV RNA templates. Replicase assays were performed with 2.5 μg of purified VP1 and 2.5 μg of BTV plus-strand RNA produced by in vitro transcription. Products were resolved by nondenaturing 9% PAGE. Lane 1, end-labeled BTV genomic dsRNA; lanes 2 to 5, products of replicase assay when performed with plus-strand RNA for BTV segments L1, L2, M4, and S10, respectively. The migration of end-labeled BTV genomic dsRNA segments L1 to S10 is indicated to the left of the image.

reaction was indeed a dsRNA, we carried out digestion with the ssRNA-specific RNase I and the dsRNA-specific RNase III. The labeled S10 product was confirmed as a dsRNA by its insensitivity to RNase I and sensitivity to RNase III digestion (Fig. 6). Furthermore, the radiolabeled material that was detected in the loading well for each replicase experiment was confirmed to be ssRNA, since it was digested by treatment with RNase I but not significantly by treatment with RNase III. Using in vitro transcripts we have found that ssRNA of more than ~600 nt does not migrate significantly into the 9% PAGE gels used in our analyses.

These results demonstrate that VP1 can synthesize dsRNA from a BTV plus-strand template in the absence of other BTV proteins or structures.

Purified VP1 initiates synthesis de novo with a plus-strand template. Our results indicated that VP1 is capable of fully processive RNA-dependent RNA polymerase (RdRp) activity. However, it was not clear whether the dsRNA synthesis detected in the replicase assay was the result of de novo initiation of RNA synthesis or of the elongation of a hairpin structure formed at the 3′ end of the plus-strand RNA. To resolve this

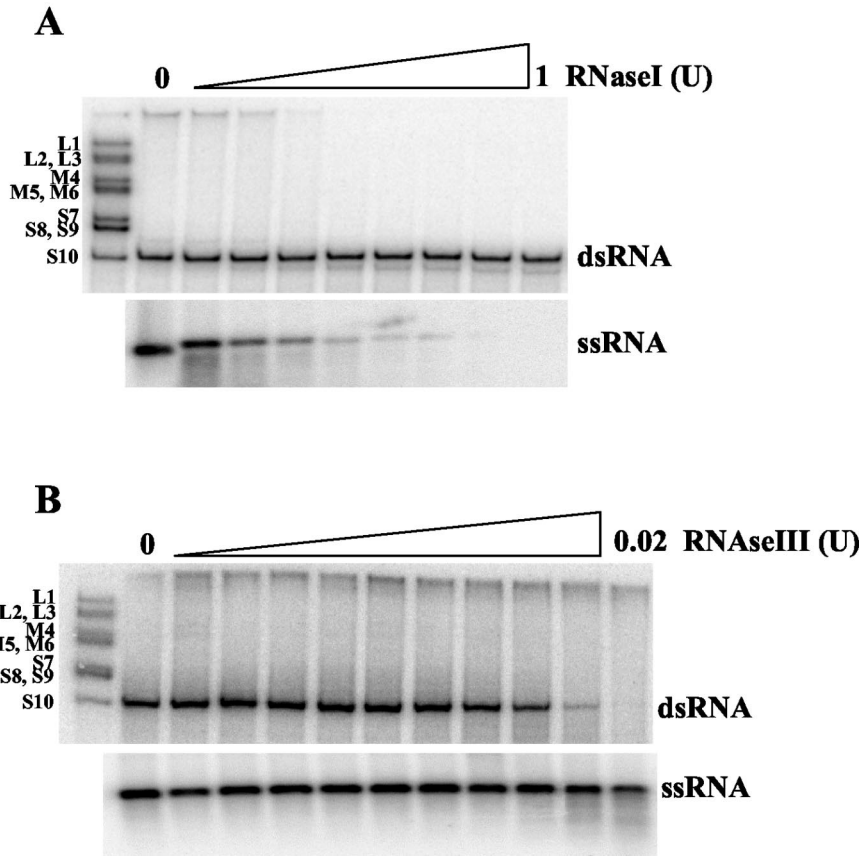

FIG. 6. The major product of the replicase assay is an RNase I-resistant, RNase III-sensitive dsRNA. (A, top panel) In vitro replication was performed with VP1 and S10 plus-strand RNA template. The product from this reaction was divided into equal aliquots and incubated with either 1 U (lane farthest to the right) or a 1:1 serial dilution of 1 U of RNase I for 1 h at 37°C. Products were resolved by nondenaturing 9% PAGE. The migration of end-labeled BTV genomic dsRNA segments L1 to S10 is indicated to the left of the image. (A, bottom panel) As a control, radiolabeled S10 in vitro transcript (ssRNA) was incubated with the same amounts of RNase I under the same conditions and resolved on a 1% agarose–formaldehyde denaturing gel. (B, top panel) Replicase products were produced as for panel A and incubated with an equivalent serial dilution starting with 0.02 U of RNase III for 40 min at 37°C. (B, bottom panel) As control S10 ssRNA was treated with the same dilution series of RNase III and resolved by 1% agarose–formaldehyde denaturing gel electrophoresis.

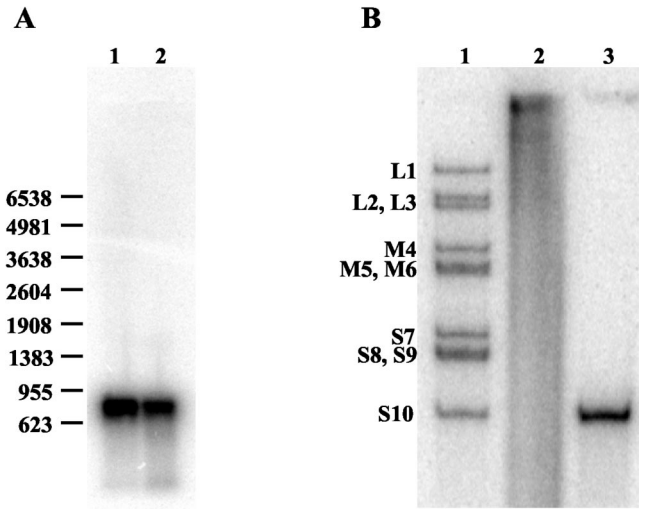

FIG. 7. Replicated RNA analyzed by electrophoresis under denaturing and nondenaturing conditions. The replicase assay was performed with VP1 and S10 plus-strand RNA generated by in vitro transcription. Replicated products were analyzed under denaturing and nondenaturing conditions. (A) Products resolved by denaturing formaldehyde agarose gel electrophoresis. Lane 1, radiolabeled in vitro transcript of S10 plus-strand RNA produced by T7 RNA polymerase; lane 2, product of VP1 replicase assay with unlabeled S10 RNA template. The migration and length in bases of ssRNA size markers on the same gel are indicated. (B) Products resolved by nondenaturing 9% PAGE. Lane 1, end-labeled BTV genomic dsRNA; lane 2, radiolabeled in vitro transcript of S10 plus-strand RNA produced by T7 RNA polymerase; lane 3, product of VP1 replicase assay with unlabeled S10 RNA template. The migration of end-labeled BTV genomic dsRNA segments L1 to S10 is indicated.

question, we separated products from the replicase assay on a formaldehyde denaturing gel. Unlabeled S10 transcript was used as a template in the replicase assay, and radiolabeled in vitro transcript of S10 plus-strand RNA was run alongside the product of the replicase assay as a marker. Under these denaturing conditions, the S10 RNA-labeled strand migrated at the same position as the labeled S10 ssRNA produced by in vitro transcription (Fig. 7A, lanes 1 and 2). Under nondenaturing conditions, the replicated segment S10 RNA migrated at the same rate as segment S10 dsRNA (Fig. 7B, lane 3), and labeled in vitro-synthesized segment S10 ssRNA largely failed to migrate into the polyacrylamide gel (Fig. 7B, lane 2). These results demonstrate that the newly synthesized strand was not produced by extension of the 3′ end of the template strand but was generated by de novo initiation of minus-strand synthesis.

Purified VP1 replicates ssRNA templates that lack the conserved 3′-end sequence of BTV plus strands. All BTV genome segments contain the conserved hexanucleotide 5′-ACUUA C-3′ at the 3′ end of the plus strand. It was possible that this sequence forms part or all of a VP1 binding site or promoter element that positions VP1 to initiate synthesis of the minus strand by using the 3′ end of the plus strand as a template. To determine whether the 3′ end of the BTV plus strand is necessary for initiation of minus-strand synthesis, segment L2 plus-strand RNAs that were extended at the 3′ end with vector sequence or truncated at the 3′ end were used as a template RNA in the replicase assay. These RNAs were generated by in vitro transcription with pL2BsmBI that had been digested with appropriate restriction endonucleases (Fig. 8A). Segment L2 plus strands extended at the 3′ end by 10 or 55 nt were replicated by VP1 (Fig. 8B, lanes 3 to 4), demonstrating that the polymerase protein would tolerate extension of the template RNA. In addition, L2 plus-strand template RNAs that were truncated at the 3′ end by 444 nt (Fig. 8C, lane 1), 863 nt (Fig. 8C, lane 2), or 2,026 nt (Fig. 8C, lane 3) were also replicated by VP1. These results demonstrate that the conserved hexanucleotide found at the 3′ end of all BTV plus-strand RNAs is not necessary for replicase activity in vitro. Furthermore, the replication of the L2 plus strand, which consists of only the first 900 nt of L2 (Fig. 8B, lane 3), shows that purified VP1 does not require the presence of any other 3′-proximal sequences normally present in L2 plus-strand RNA.

DISCUSSION

We describe here a processive replicase activity associated with soluble BTV VP1 in the absence of other viral proteins. The replicase activity of purified recombinant VP1 has a optimal Mg^{2+} concentration similar to that previously determined for the optimal transcriptase activity of intact BTV core particles (33, 34). This supports the hypothesis that there is no major change in polymerase activity caused by dissociation of VP1 from the rest of the core complex. In addition, whereas replicase activity in vitro was low, enzyme activity was detectable for at least 24 h at 37°C (Fig. 4), and the rate of this reaction was essentially constant for the first 4 h of this incubation. VP1 was not only able to copy the smallest BTV RNA (822 nt) fully but also able to synthesize several larger plus-strand BTV RNAs of different sizes, including the largest BTV plus-strand RNA of 3,954 nt (Fig. 5). We confirmed that the products of these reactions were RNase I-resistant, RNase III-sensitive dsRNAs. Interestingly, in all of our replicase experiments we noted a significant amount of RNase I-sensitive, RNase III-resistant radiolabeled material that failed to migrate far into the 9% PAGE gels used to resolve dsRNA products (Fig. 6). This is consistent with the migration of radiolabeled in vitro transcripts on these gels (Fig. 7B). We suggest that the most likely explanation for this material is that it is either partial duplex RNA formed as replication intermediates or that it is ssRNA produced by de novo initiation on the newly synthesized minus strand. In this assay newly synthesized, radiolabeled minus strand migrates at the same position as in vitro transcript for the same BTV plus-strand RNA (Fig. 7A). This suggests that the minus-strand product is formed by de novo initiation rather than extension of a terminal hairpin-like structure in the template RNA.

Surprisingly, given the well-documented template specific binding of the polymerase of rotavirus (18–20, 31), another member of the *Reoviridae*, BTV VP1, replicated template RNAs that did not contain the conserved terminal hexanucleotide common to all BTV genome segments. This was also true for templates that had termini that bear no resemblance at all to these conserved sequences, although our preliminary analyses suggest that the polymerase does have a preference for templates with a terminal C nucleotide (data not shown). Interestingly, the reovirus λ3 polymerase, which has been shown to be a poly(C)-dependent poly(G) polymerase (28), is also

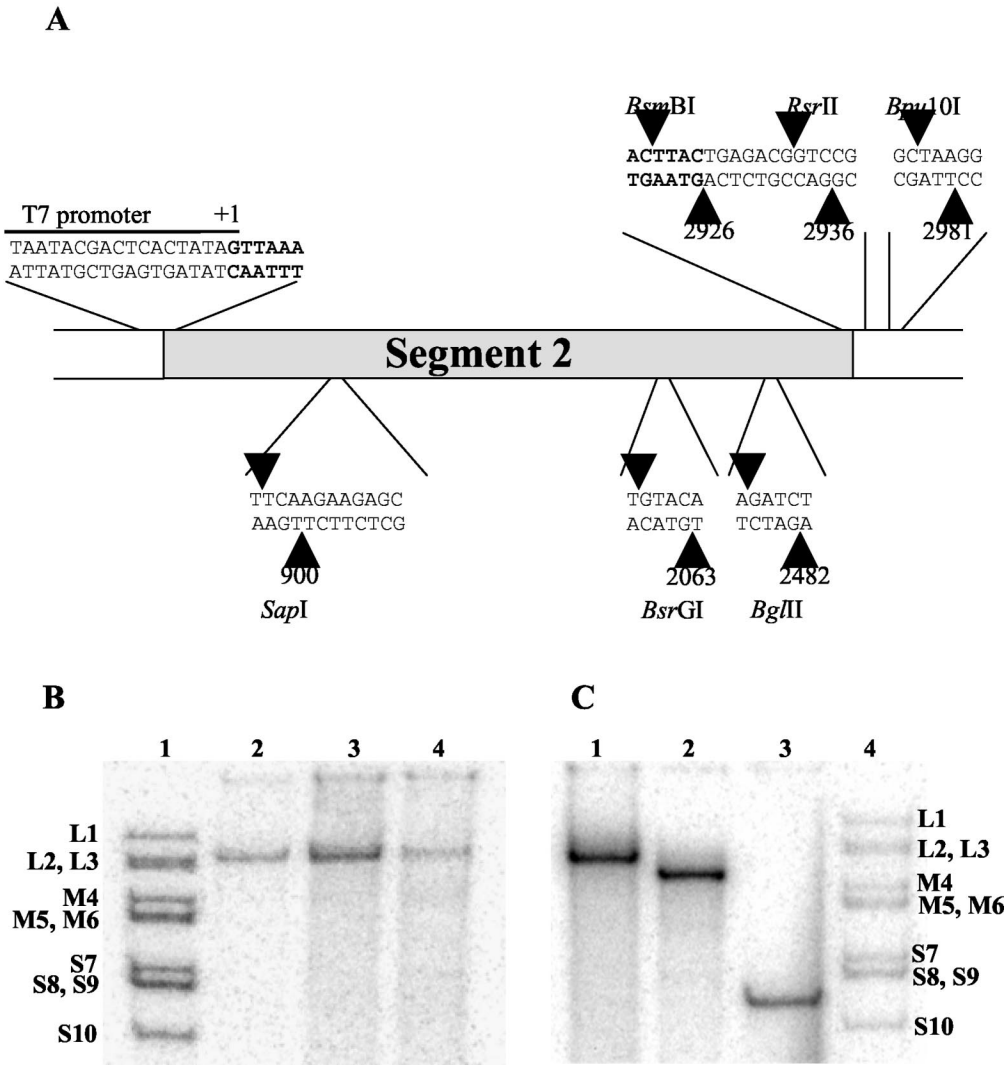

FIG. 8. In vitro replication of 3′ extended and 3′ truncated L2 plus-strand RNA. Template RNA for the replicase assay was produced by in vitro transcription of the construct shown in panel A after linearization with the restriction endonucleases indicated. BTV sequences are indicated in boldface. Restriction endonuclease cleavage sites are indicated with arrows, and the length in nucleotides of each in vitro transcript is indicated. In vitro replication was performed with 2.5 μg of purified VP1 and 5 μg of BTV plus-strand RNA generated by in vitro transcription. Products were resolved by nondenaturing 9% PAGE. (B) Gel showing the replication of BTV segment L2 plus-strand RNAs extended at the 3′ end with vector sequence. Lane 1, end-labeled BTV genomic dsRNA; lane 2, product of the replicase assay with BTV full-length L2 plus-strand RNA; lanes 3 and 4, product of the replicase assay with L2 plus-strand template extended by 10 and 55 nt, respectively. (C) Products from the VP1 replicase assay with truncated L2 transcripts. Lanes 1, 2, and 3, replicase products with L2 templates truncated by 444, 863, and 2026 nt, respectively. The migration of end-labeled BTV genomic dsRNA segments L1 to S10 is indicated.

capable of initiating dsRNA synthesis on a nonviral template in the absence of other viral and cellular proteins (30). Thus, BTV and reovirus polymerases show some characteristics that are quite distinct from those of rotavirus, which has a polymerase that is only active in the presence of the VP2 subcore protein (20, 31).

All of the transcripts that were used as templates in our assays were produced by using T7 RNA polymerase with a DNA template. We note that previous studies with T7 polymerase have suggested that, although the majority of transcripts are full length, there is a minority of transcripts that are extended by a single nucleotide (10, 17). Since the same T7 polymerase system has been used to produce template in the rotavirus "open-core" system it is unlikely that the apparent lack of specificity for BTV VP1 is solely the result of extension of some templates by the same nucleotide usually found at the 3′ terminus of BTV plus-strand RNA. In our hands, the efficiency with which RNA template was used by VP1 was unaffected by whether the DNA template for in vitro transcription by T7 polymerase had a blunt end or a 5′ overhang in the minus strand (data not shown).

The BTV replicase activity appears to be much more similar to what has been reported previously for the dsRNA bacteriophage ϕ6 (13, 15). However, the activity of VP1 as a replicase protein in the absence of other viral components does present a potential problem for the biology of the virus. It is difficult to

explain how a virus that has developed an elaborate transcriptionally active core particle to contain a dsRNA genome could tolerate promiscuous initiation of dsRNA synthesis in virus-infected eukaryotic cells by a constitutively active polymerase protein. We note that in our assays the RdRp activity associated with recombinant BTV VP1 is low, with a small minority of potential template molecules being replicated. This low RdRp activity of VP1 alone may be one mechanism by which BTV avoids synthesizing free dsRNA in the cytosol. Other explanations for the low activity of recombinant VP1 include the possibilities that a fraction of the purified VP1 may be misfolded or inactivated during purification or that other viral proteins normally act to modulate the activity of VP1 in the assembling core particle. Indeed, as has been shown in vitro with hepatitis C polymerase (21, 27), the evidence from other viral replicase systems seem to suggest that polymerase activities are highly regulated in vivo. In the case of BTV, such modulation may take the form of inhibition of the replicase activity of soluble VP1 in the cytosol in order to prevent the formation of free dsRNA. Template specificity and/or improved efficiency of initiation of replicase activity may be provided by other viral proteins present in the assembling virus core. A similar effect on RdRp activity has been noted in the reconstituted influenza virus replicative transcription complex (12).

We cannot rule out, on the basis of the recent findings regarding the importance of the rotavirus subcore protein VP2 to the initiation of dsRNA synthesis on a plus-strand template (20, 31, 36), the possibility that the BTV subcore plays some role in viral genome replication. It seems unlikely that there is a fundamental difference in the way that core assembly and genome replication occur in different genera of the family *Reoviridae*. However, it is clear from the present study that for BTV the polymerase protein alone is sufficient for initiation and processive elongation of dsRNA on an ssRNA template. The apparent discrepancy may be due to differences in purification procedures of the different proteins, the enzymatic stability of individual polymerase proteins, and the sensitivities of the different experimental systems used to assay RNA synthesis activity. It will be interesting to determine whether the BTV VP3 subcore protein has any role in either enhancing the specificity or the rate of initiation of VP1 synthesis on a plus-strand template; this will no doubt be an area for future investigation.

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