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Characterization of Double-Stranded RNA Satellites Associated with the *Trichomonas vaginalis* Virus

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Three small and distinct satellite double-stranded RNAs (dsRNAs) denoted s1, s1′, and s2 were recently described for a *Trichomonas vaginalis* isolate harboring a dsRNA virus. Since characterization of these satellite dsRNAs might provide insight into the virus replication cycle and virus-host interactions, full-length cDNAs to s1 and s1′ dsRNAs were synthesized and sequenced. s1 dsRNA has 688 bp, and s1′ dsRNA has 616 bp. A 228-bp open reading frame that begins at nucleotide 37 was detected on a putative sense strand of s1. All satellite RNAs were found associated with RNA-dependent RNA polymerase (RDRP) activity that banded on CsCl gradients. Within carrier trichomonads, satellite RNAs synthesized single-stranded replicative intermediates. An in vitro assay was established to assess replication of satellite RNAs. Transcripts generated from s1 cDNA, for example, served as a template for the viral RDRP. These templates had a polarity similar to that of the replicative intermediate found in the satellite-harboring parasites. Importantly, the recognition of s1 RNA was shown to be specific, since unrelated RNAs did not serve as templates for RDRP under the same experimental conditions. The data indicate that the cDNA of s1 has a specific and essential sequence needed for recognition by the viral RDRP and for subsequent RNA synthesis. Both s1 and s1′ have conserved domains, albeit of unproven function, but which may be required for replication.

Numerous *Trichomonas vaginalis* clinical isolates are persistently infected with double-stranded RNA (dsRNA) viruses (13, 24). Three segments (L, M, and S) that are constitutively replicated in the virus-harboring isolates have been identified (13). These segments did not cross-hybridize (13) and were associated with virus particles possessing an RNA-dependent RNA polymerase (RDRP) activity (16). A recent report has presented the nucleotide sequence of a nonsegmented dsRNA genome of another virus from *T. vaginalis* (22). The relationship between this dsRNA and the virus(es) containing three dsRNA segments is unknown.

A role for the *T. vaginalis* virus with the three dsRNA segments on trichomonal biology has been established. The presence and replication of the viral dsRNAs are related to the property of phenotypic variation for a prominent immunogen (P270) among infected trichomonads (25). This property was absent in all isolates without the virus as well as in progeny trichomonads that lost the virus (14, 25). In virus-harboring parasites, transcription of the phenotypically varying P270 gene was upregulated (14), resulting in elevated amounts of P270 and showing that the presence of virus influences expression of this and other trichomonad proteins, including cysteine proteinases (15, 19).

Upon characterization of the virus RDRP, several small dsRNAs were identified (16). These mini-replicons have properties resembling satellite RNAs associated with plants and yeast (*Saccharomyces cerevisiae*) RNA viruses (12, 18, 27). The small dsRNAs do not cross-hybridize and are unrelated to either the viral genomes or any nucleic acid from noninfected trichomonads (13, 16). These mini-replicons were not detected in *T. vaginalis* isolates without the virus. The fact that synthesis of these small RNAs is simultaneously abolished upon elimination of the viral genome (16) suggests that these dsRNA species depend on the virus for propagation. Furthermore, the small RNAs were associated with viral RDRP activity and were purified by CsCl gradients. Finally, these mini-replicons are not required for virus replication, because some virus-infected *T. vaginalis* isolates do not possess these elements (16). Amplification of the small dsRNAs in virus-infected trichomonads was temporal, occurring only under stressful cultivation conditions (16). We propose that these newly discovered mini-replicons are satellite dsRNAs associated with *T. vaginalis* virus. The significance of amplification of the satellite RNAs is unknown.

We reasoned that characterization of the dsRNA satellites might provide insight into the biology of the *T. vaginalis* virus. Full-length cDNAs were obtained for dsRNAs s1 and s1′, and the nucleotide sequences were determined. The satellite RNAs were associated with particles having RDRP activity. Within the infected parasites, full-length single-stranded RNAs (ssRNAs) were identified as replicative intermediates. An in vitro assay was established for the replication of s1 satellite RNA by using cloned cDNA. This report provides a basis by which the satellite RNAs can serve as a model for studying the replication of *T. vaginalis* virus and for identifying the *cis*-acting elements essential for viral RNA synthesis.

MATERIALS AND METHODS

Cultures. The conditions for cultivation of *T. vaginalis* isolate T068-IIc1 in batch or chemostat cultures were described recently (13, 16).

Cloning and nucleotide sequencing of s1 and s1′ cDNA. s1 and s1′ were separated from total dsRNA by electrophoresis in low-melting-point agarose gels (13, 16). Approximately 1 μg of satellite dsRNA was polyadenylated in vitro with *Escherichia coli* poly(A) polymerase (GIBCO BRL, Gaithersburg, Md.) according to the manufacturer's instructions. The average length of the poly(A) tail was determined by sequencing to be around 16 bases. The poly(A)-tailed RNA was denatured by boiling for 3 min and was reverse transcribed in a reaction buffer (50 mM Tris-HCl [pH 8.3]; 40 mM KCl; 6 mM $MgCl_2$; 1 mM dithiothreitol; 0.5 mM [each] dATP, dCTP, dGTP, and TTP; 0.1 mM oligo(dT); 0.1 mg of bovine serum albumin per ml; and 24 U of avian myeloblastosis virus reverse transcriptase [GIBCO BRL]) and incubated for 40 min at 50°C. The RNA-DNA

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hybrid was treated with 1 U of ribonuclease H at 12°C for 1 h. Second-strand DNA synthesis was carried out in the presence of 115 U of DNA polymerase I and with incubation at 22°C for 1 h. The reaction was stopped by incubation at 70°C for 10 min, and the cDNA was blunt ended with 1 U of T4 DNA polymerase at 37°C. The purified cDNA was cloned into the *Sma*I site of linearized pGEM4Z (Promega Corporation, Madison, Wis.) cloning vector. The recombinants were transformed into competent *E. coli* JM109 cells. Positive clones were selected by insert analysis and confirmed by Northern (RNA) analysis. Two clones labeled pVss18 and pVss10, for the s1 and s1′ satellite RNAs, respectively, were used for the experiments described in this paper.

The nucleotide sequence determination was performed twice for both strands of the cDNA clones by the dideoxy-chain termination method (21) with automated DNA sequencing. Sequence analysis was performed with PC Gene computer software.

Northern analysis. Total RNA from infected trichomonads was obtained by the methods of Chomczynski and Sacchi (2) as previously described (13). ssRNA was separated by treatment of total RNA with 2 M LiCl and incubation at 4°C for 4 h, followed by centrifugation at 10,000 rpm for 10 min in a microcentrifuge (20). Approximately 10 μg of ssRNA was electrophoresed into a denaturing 1% agarose gel and blotted onto a Zeta probe nylon membrane (Bio-Rad Laboratories, Inc., Richmond, Calif.) in the presence of 50 mM NaOH in accordance with the manufacturer's instructions. Prehybridization was carried out for 2 h in 50% formamide–100 mM Na_2PO_4 (pH 7.0)–125 mM NaCl–7% sodium dodecyl sulfate (SDS) at 55°C. Hybridization was carried out in a similar solution containing $\sim 10^8$ cpm of radiolabelled riboprobes generated from the s1 and s1′ cDNA clone linearized with *Bam*HI and transcribed with SP6 RNA polymerase. Filters were washed sequentially for 30 min each time in 2× SSC (1× SSC contains 150 mM NaCl and 15 mM Na_3-citrate [pH 7.0])–0.1% SDS, 0.5× SSC–0.1% SDS, and 0.1× SSC–0.1% SDS at 55°C before the blots were exposed to X-ray films.

Virus purification and RDRP. Virus particles were obtained as recently detailed (13, 16). Briefly, 4×10^9 trichomonads cultivated under chemostat conditions (16) were suspended in TNM buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 5 mM $MgCl_2$) and lysed by sonication. The lysate was clarified by being centrifuged twice at 10,000 × *g* for 20 min in a Sorvall SS34 rotor. The supernatant was then pelleted through a 20% sucrose cushion prepared in TNM buffer at 100,000 × *g* in an SW40 rotor for 2 h. The sediment containing the virus particles was suspended in TNM buffer, equilibrated to a density of 1.35 g/ml with CsCl, and recentrifuged at 100,000 × *g* for 24 h. Twelve 1-ml fractions were collected from each tube and diluted in 24 ml of TNM buffer. Virus particles were pelleted by centrifugation at 100,000 × *g* for 2 h and used in the polymerase assay.

For generation of active replicase, the virus material present in the gradient (see Fig. 3, lane 10) was dialyzed in a low-ionic-strength buffer (1 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 5 mM 2-mercaptoethanol) in order to obtain empty particles (1, 7). The released RNA was removed by centrifugation at 100,000 × *g* on a 10 to 40% continuous sucrose gradient made in TNM buffer in an SW40 rotor for 2 h. The empty particles were harvested from the top gradient fractions (3–5), diluted in 12 volumes of TNM buffer, and pelleted by centrifugation as described above. Particles devoid of any detectable viral RNA but containing active RDRP were also obtained from a cytoplasmic fraction treated with the low-ionic-strength buffer, followed by centrifugation at 100,000 × *g* in the CsCl gradient as described above. Fractions of the gradient with a density of ~1.28 to 1.30 were harvested and diluted in TNM buffer. The polymerase complex was then pelleted by centrifugation as described above.

Polymerase activity of virus particles was assayed as reported previously (6, 16). Briefly, pelleted viral complexes from different fractions of the CsCl gradients were resuspended in 100 μl of buffer A (50 mM Tris-HCl [pH 7.5]; 20 mM NaCl; 5 mM $MgCl_2$; 0.1 mM EDTA; 5 mM KCl; 1 mM [each] ATP, GTP, and UTP; 50 μCi of [α-^{32}P]CTP; and 50 U of RNasin), with or without 50 μg of actinomycin D per ml. Reaction mixtures were incubated at 37°C for 1 h and stopped by addition of 5 mM EDTA–0.5% SDS. Nucleic acids were extracted by phenol-chloroform and precipitated in the presence of 1 volume of 4 M ammonium acetate and 3 volumes of ethanol (20). Products were analyzed by agarose gel electrophoresis and autoradiography.

For in vitro replication assays, s1 transcript was generated from the s1 cDNA clone (pVss18), linearized with *Eco*RI or *Bam*HI, and transcribed with T7 or SP6 RNA polymerase, respectively. The integrity of the synthesized transcripts was assessed on a 1.5% agarose gel. Different quantities of RNA of opposite polarities (as indicated in the figures) were added to the reaction mixture. Incubation and processing were performed as described above.

RNase protection analysis. s1 transcripts replicated in vitro were treated with RNase T_1 (50 U/ml) in the presence of 50 or 500 mM NaCl and with incubation at 37°C for 30 min. The reactions were stopped by addition of 5 μl of 500 mM EDTA. The products were extracted with phenol-chloroform and precipitated with ethanol. Analysis was by agarose gel electrophoresis and autoradiography.

Nucleotide sequence accession number. The nucleotide sequences s1 and s1′ have been assigned GenBank accession numbers U30166 and U30167, respectively.

A.

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                                        *
5'- CAGAGATATCGGTGCAAAACCTGTACTGTTCCGCTCATGTCAAGATTGTG     50
    CTCGGGCGCTACCCGTTACCAATCAGACATGTGGTTTATTTCACCTACCC    100
    CACGGCAAGGCAGGACACAGAGTACCCCTGAGACGCACAGACATATGCAT    150
    TATCCCTACTATCTGCGACACTTACTCTTCGTCACCCACAATGACGTCTA    200
    CAAAATGCTCAATGTAAGACCAACAACCAAGAGCCAGCTTACGCCCGCCC    250
    ATTATACTTGCTTTTAAAGCGGGAGTTATGATTGGCGGGATAAAGAACAT    300
    AAGTAGCAAAAGAGTAAACATCGCCATGAATAGTACGGCTCAACACCTAG    350
    GACAACGTTAAGTCTAAGTAAAGCAGGTGGGTGGTTGACGAGACATCCAG    400
    TACCTAGAACTCAACCAGATGCCTCCCCCCTGTATTACTTCACTATTCAA    450
    AAGCAGGTATAGCATTCCCATACCACAGACTGCTCGATGTGAGTCGACCA    500
    GAGAGGGCGCTGTACGAACCTGAACTATAGACGCGCTCCAGCATAGGCTG    550
    GCCAAAAAAGGCTCATTCTTCCTCACAGAAGAATGCATTACCGCCTATCC    600
    CGGGGCTACGTCAGATAGTTGGTGCCGGGACACTCGTATATGGTTTACAC    650
    CGATATACACTTAAAGACTTCCTATCGCTTCTTTAAGC - 3'           688
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B.

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5'- CGTGTTCAGGGACGTTGCCACAAGCACGGGTCGTACCTCCCATCCTTAGC     50
    GGTTTTGACCAACTACAAGGACACGCATCCGAGACAGAGCTCCTGCGTCC    100
    AGGTATTAGCCCGCTATCTAACACGCTAGCGTTTTAGAGACTGACGTCAA    150
    TAGCCTTTACCTACGCAAACAGAGCCCCCTCTTCAACATGCCCACATTGA    200
    TCTCAACGAGCCGGGGTACCGACATGGGACTTCACTCTCACCGCGTCATT    250
    ACGACCTGTAGGTACCTATTACAGTAACGTCTGGGTCTAGCACTCGGTTT    300
    GAACCAAGATTCGCTATGGGTCGGCAGTCGACTCGGGGTGAGCAGTCCCG    350
    AGGCCCCCTTTTGCGCAACGCTGTCCGTAGTTTCGTGATACAGGCGATGT    400
    ACTGTGCCCCTAACCCCCGTCCGCCCCATTGCGCCGAACCTCGGTAGCTT    450
    TCGACTCTGTACCCTAATACTACGACACATACCCAGAAAGCGATTTCACC    500
    TCATTCTTCCGCAGTAGAGAGATTCCATTGGATGTAGGTGCCCATACATT    550
    ACGTATGAGAATACTACCGGATACGCGACGCCCGGTAGGCCTAAAAATCA    600
    TTTCAGGTTTTTAAGC - 3'                                 616
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FIG. 1. cDNA cloning and nucleotide sequencing of s1 and s1′ satellite dsRNAs. cDNAs were obtained from gel-purified, in vitro-polyadenylated s1 and s1′ dsRNAs. Detailed procedures for cloning and sequencing are outlined in Materials and Methods. (A and B) Nucleotide sequences of the putative sense strands of cDNAs for s1 (A) and s1′ (B). Two conserved domains among the two sequences are underlined, as described in Results. The potential ORF on s1 genomes is identified by an asterisk.

RESULTS

Molecular cloning and nucleotide sequencing of s1 and s1′. Full-length cDNAs of s1 and s1′ satellites were obtained from the in vitro-polyadenylated dsRNAs. Nucleotide sequencing of the generated cDNAs revealed that s1 dsRNA contained 688 bp and s1′ had 616 bp (Fig. 1). The ends for each segment were confirmed by sequencing of cDNA clones that were polyadenylated at the 3′ ends of the opposite strands. The sizes of the cDNAs were consistent with those estimated from agarose gels (16). The s1 and s1′ dsRNAs contained G+C contents of 48 and 52%, respectively. The 7 nucleotides at the 3′ end of both s1 and s1′ contained the sequence 5′-TTTAAGC-3′ (double underlined in Fig. 1). A second conserved domain with the nucleotide sequence of 5′-CTCATTCTTCC-3′ (underlined in Fig. 1) was also present at ~100 nucleotides upstream of the 3′ end of both s1 and s1′. A potential open reading frame (ORF) (asterisk) on the putative sense strand of s1 begins at nucleotide 37 and terminates at nucleotide 264. A scan of databases at the nucleotide and amino acid levels revealed no homology with known sequences for the putative protein encoded by the potential ORF. No similar ORF was apparent for s1′.

Satellite RNAs replicate through a full-length single-stranded intermediate. We next attempted to characterize replicative intermediates of satellite RNAs and to determine whether their synthesis was also affected by stressed environments. Northern analysis was done with ssRNA obtained from batch and chemostat cultures of infected trichomonads by using the putative antisense probes of s1 (Fig. 2A) and s1′ (Fig. 2B). A full-length, single-stranded intermediate for each of the satellite RNAs was seen amplified only in parasites cultivated in the chemostat at extended generation times of 24 and 48 h (lanes 2 and 3, respectively) compared with similar amounts of

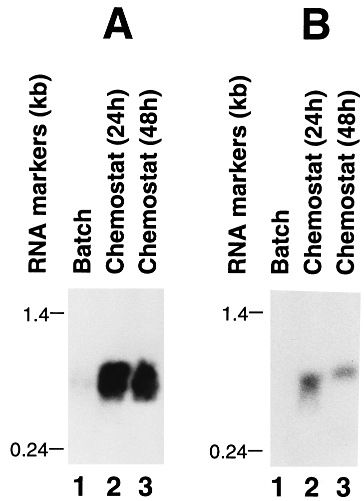

FIG. 2. Analysis of ssRNA of infected trichomonads for satellite transcripts. Approximately 10 μg of ssRNA of infected trichomonads was analyzed by Northern analysis. Anti-sense riboprobes were generated with the SP6 promoter from the cDNA of s1 (A) or s1′ (B) cloned into pGEM4Z. RNA in lane 1 was obtained from trichomonads from batch cultures, as described previously (13). Lanes 2 and 3 contained RNA obtained from trichomonads cultivated in the chemostat with generation times of 24 and 48 h, respectively. ssRNA size markers are shown on the left (GIBCO-BRL).

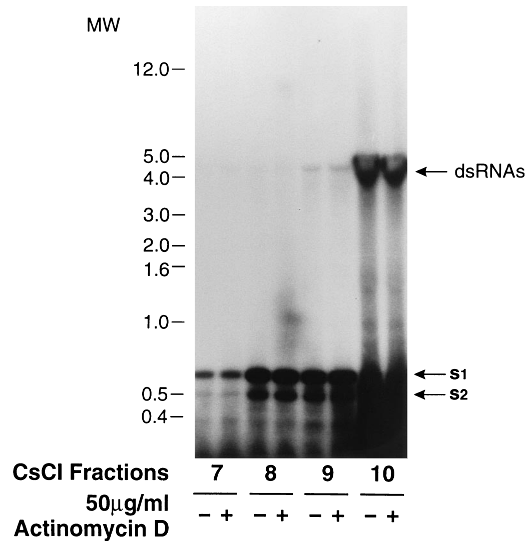

FIG. 3. Evidence for association of satellites with active RDRP. Particles sedimented through a 20% sucrose gradient were fractionated on a CsCl gradient. Different fractions were centrifuged, and pellets were assayed for the presence of RDRP activity (Materials and Methods) (16). The majority of virus particles banded in fraction 10, as determined by polymerase activity and as shown previously (16). Satellite-RDRP complexes were primarily present in fractions 7 to 9. Analysis of the polymerase products was by electrophoresis in a 1% agarose gel and by autoradiography. dsRNA refers to the viral genome, and s1 and s2 indicate the migration of satellite RNA as determined previously (16). Actinomycin D (50 μg/ml) was included in the reaction mixture (lanes labeled +). Relative positions of molecular size markers (MW [kilobase pairs]; GIBCO BRL) are shown on the left.

ssRNA obtained from batch-grown trichomonads (lane 1). Interestingly, under identical conditions, s1 transcripts were present in larger quantities than s1′ transcripts on the basis of the intensity of the bands, indicating differential amplification of the satellites. The opposite sense ssRNA strands were not detected (data not shown). These data demonstrate the presence of a full-length ssRNA intermediate for the s1 and s1′ RNAs and showed that upregulation of transcription of satellite RNAs occurred under stressed conditions.

Association of satellite RNAs with active RDRP. To better understand the mechanism of satellite RNA synthesis, in vitro polymerase assays were performed with CsCl gradient fractions containing the satellite RNAs. As shown in Fig. 3, fraction 10 contained the majority of the virus particles with dsRNA segments (16), showing the predominant synthesis of viral dsRNA (arrow). Satellite RNA and RDRP complexes also banded in fractions 7 through 9 and, upon incubation in vitro, synthesized the small dsRNAs. In RNase T_1 protection assays, these polymerase products resisted degradation under high-salt conditions (data not shown), consistent with a double-stranded nature. The polymerase activity responsible for the synthesis of satellite RNAs was not affected by the presence of actinomycin D, suggesting that an RDRP was associated with satellite RNAs. Similar experiments performed in trichomonads without the dsRNA virus (13, 16) showed no evidence of RDRP activity and satellite dsRNAs. These data indicate that satellite dsRNAs associate with and are propagated by an RDRP in the virus-harboring trichomonads.

Replication of s1 transcripts by viral polymerase. Attempts were made to establish an in vitro replication assay for the satellite dsRNAs associated with the virus. In these studies, s1 RNA was used as a template. Single-stranded transcripts of opposite polarities were generated from the cloned cDNA of s1 and used in polymerase preparations obtained from the virus particles (Materials and Methods). The polymerase in these preparations recognized as templates the in vitro-generated s1 transcripts with polarity similar to that found in the infected trichomonads. Figure 4A (lanes 4 through 6) shows the replication products of these assays (arrows). The products were heterogeneous in length, suggesting the incomplete replication of some templates. The antisense strand of s1 (Fig. 4B), brome mosaic virus RNA, and yeast tRNA did not serve as templates for RDRP. Attempts to use the s1 dsRNA as a template were not successful. It is important to note that the in vitro-generated transcripts contain 25 bases of the vector and an additional 16 thymidine bases at the 5′ end because of the in vitro polyadenylation for cDNA synthesis. Although the effect of these additional sequences on the in vitro replication of s1 is unknown, the ability to detect replication products shows that s1 is a template for RDRP.

The RDRP preparations described above contained residual viral RNA, as demonstrated by generation of small RNAs in reaction mixtures with and without exogenous templates. An active replicase preparation devoid of any detectable viral RNA was obtained by direct purification of particles in a low-ionic-strength buffer (1). No polymerase activity was observed in the absence of any added RNA (Fig. 5A, lane 1) or unrelated RNAs, such as BMV genomes or yeast tRNA. On the other hand, polymerase activity was detected with small amounts (0.1 μg) of in vitro-generated transcripts of s1 satellite RNAs added to the reaction mixture (Fig. 5A, lanes 3 through 6). The polymerase products migrated in agarose gels and had the size of the s1 dsRNA detected in the infected trichomonads (16). These data suggest that the single-stranded intermediate of s1, detected in the infected trichomonads, can serve as a template for viral RDRP. Furthermore, the s1 satellite RNA contained the nucleotide sequence(s) essential for viral polymerase recognition and subsequent replication. Finally, Fig. 5B shows that the in vitro-replicated s1 products were resistant to RNase T_1 degradation under high concentrations of salt but were degraded in reaction mixtures containing low concentra-

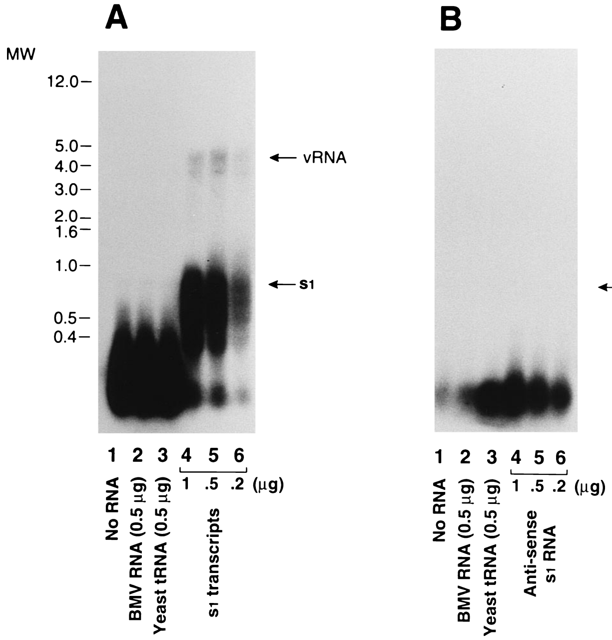

FIG. 4. Replication of s1 transcripts in vitro by RDRP associated with the virus particles. The RDRP preparations were obtained by treatment of virus particles derived from fraction 10 in Fig. 3 with a low-ionic-strength buffer (Materials and Methods). Viral RNA (vRNA) was removed by centrifugation of ruptured particles in a sucrose gradient. Fractions were assayed for the ability to replicate the s1 exogenously added transcripts as described in Materials and Methods. Single-strand forms of s1 were obtained from cDNA clones linearized with *Bam*HI or *Eco*RI by SP6 and T7 RNA polymerase, respectively. (A) Increase in replication products when different amounts of transcripts generated from the T7 promoter were added (lanes 3 to 6). (B) Putative antisense s1 RNA was not replicated by these preparations. In lanes 1, no RNA was added. Lanes 2 contained yeast tRNA (0.5 µg [GIBCO BRL]), and lanes 3 had 0.5 µg of brome mosaic virus (BMV) RNA (Promega). Molecular size markers (MW [kilobase pairs]) are the same as for Fig. 3.

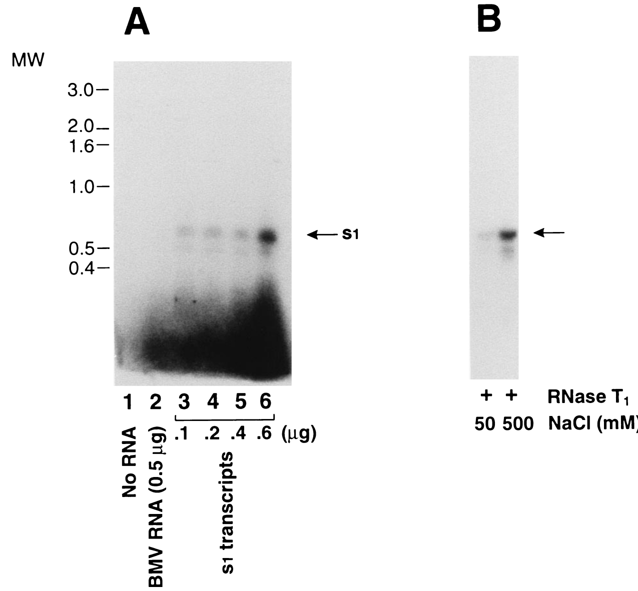

FIG. 5. Replication of the s1 satellite by soluble RDRP activity obtained from CsCl gradients. RDRP preparations were obtained by fractionation of infected lysate on CsCl gradient in low-ionic-strength buffers. In vitro replication assays were as described in the legend to Fig. 4. (A, lanes 3 to 6) Replication of s1 transcripts generated from cDNA by RDRP. The arrow shows the migration of completely replicated products. BMV, brome mosaic virus. (B) In vitro-synthesized products are resistant to RNase T_1 in high concentrations of salt. Molecular size markers (MW [kilobase pairs]) are the same as for Fig. 3.

tions of salt. This is consistent with the double-stranded nature of the polymerase products and the association of an active replicase with these preparations.

DISCUSSION

Previous studies in this laboratory indicated the presence of several distinct satellite dsRNAs associated with some *T. vaginalis* isolates infected with the dsRNA virus (16). In this report, cDNA cloning of two of the satellite dsRNAs was accomplished and their nucleotide sequences were determined. The findings presented here also demonstrated amplification of full-length single-stranded replicative intermediates of the satellites under stressed environments. Furthermore, an in vitro replication assay instrumental in the characterization of these conditionally synthesized mini-replicons was established.

Although the s1 and s1′ dsRNAs were different in size and sequence, common motifs conserved for both were apparent. In addition, but perhaps not unexpectedly, similar configurations were observed in the secondary structures of the putative sense RNAs as determined by computer-assisted folding (data not shown). These secondary structure predictions are not unlike those shown to exist for the yeast L1 segment virus-binding site and the S14 segment sequence necessary for interference with M1 replication or packaging (10). The biological significance of these conserved domains and secondary structures is unknown and awaits further experimentation. The sequence differences of these satellite RNAs may be responsible for distinct biological properties, albeit to be determined, or differential amplification of ssRNA as observed in these studies (Fig. 2). This idea has had precedents, because the satellite RNAs associated with cucumber mosaic viruses, which have different nucleotide sequences, display a wide range of biological activities, such as disease induction or attenuation (12, 18, 23). Considering that the presence of *T. vaginalis* virus is associated with phenotypic variation and upregulation of a major cellular immunogen (14), it is important to determine whether amplification of the satellite RNAs has any functional significance to the *T. vaginalis* virus or to the biology of the parasite.

Several lines of evidence suggest that the satellite RNAs depend on the parent virus for replication. The results of the polymerase assays demonstrated that satellite RNAs interact with and use RDRP for propagation. Neither the satellite RNAs nor any RDRP activity has been detected in trichomonads without detectable virus. In addition, the nucleotide sequences of the satellites do not show any potential ORF encoding RDRP. Furthermore, RDRP preparations obtained from the virus particles facilitated the replication of s1 transcripts in vitro. It is noteworthy that ssRNAs of s1 were specifically recognized and replicated by the viral RDRP. All of these data are consistent with the idea that s1 and s1′ satellites depend on the dsRNA virus for replication.

The observation that nonviral templates from unrelated viruses are not recognized by the polymerase preparations supports the notion that specific nucleotide sequences or secondary structures dictate recognition by the polymerase for RNA synthesis. Interestingly, two common motifs were detected at the 3′ ends of the sequences of both s1 and s1′. The same motifs were not present in the sequence of a nonsegmented dsRNA genome of another virus from a *T. vaginalis* isolate (22). However, since most virus-harboring *T. vaginalis* isolates have multiple dsRNA segments (13), it will be of interest to know whether any of the segments, especially those not represented by the nonsegmented genome of the recent report (22), possess these motifs or secondary structure predictions. *cis*-acting domains essential for viral RNA replication by RDRP have been identified for several viruses. For instance, in addition to an internal site, nucleotides at the 3′ end of the positive strands of the yeast (*Saccharomyces cerevisiae*) L-A dsRNA virus are required for optimal replication in vitro (4, 5). Similarly, the noncoding nucleotide sequence at the 3′ end of poliovirus genome has been shown to contain *cis*-acting domains essential for RNA amplification (11). The availability of an in vitro replication assay, along with cDNAs to satellite dsRNA, allows further investigations of the mechanism of RNA replication by *T. vaginalis* virus. It may be possible in the future to demonstrate whether these sequence motifs are essential for interaction with the viral RDRP.

A step essential in the replication of all dsRNA viruses is the synthesis and simultaneous packaging of a ssRNA intermediate (27). Although the level of dsRNA replication for *T. vaginalis* virus is relatively high during normal replication (14), the ssRNA intermediates for these genetic elements are present in low copy number and are detectable only by PCR amplification (15). The reason for this asymmetric viral RNA synthesis is unknown. On the other hand, the ssRNA intermediates for the s1 and s1′ satellites were relatively abundant under stressful chemostat conditions involving extended generation times and nutrient limitation. Thus, preferential transcription of these satellite RNAs may be controlled by environmental cues. This may be analogous to amplification of 23S and 20S RNAs detected in yeast cells in stress environments of heat and starvation (17, 26). What accounts for the different levels of s1 and s1′ transcripts is unknown. It is possible that the sequence variation among the satellites imposes a different affinity for viral replicase or other factors essential for amplification. The ssRNA intermediates identified for s1 and s1′ are likely the equivalent of positive sense RNAs identified in other dsRNA viruses (27) and, as demonstrated here for s1 (Fig. 4 and 5), serve as a template for synthesis of double-stranded forms. At this point, whether satellite RNAs of *T. vaginalis* are encapsidated by viral proteins is unclear. However, on the basis of in vitro polymerase assays, it is evident that RNAs of these satellites are at the very least associated with the viral RDRP, and possibly other essential viral and/or cellular factors (27), leading to the synthesis of ssRNA and dsRNA satellites. Alternatively, the mechanism of replication for s1 and s1′ satellites may resemble the T and W dsRNA species of yeast strains in which the interaction between the dsRNA and RDRP occurs without any evidence for encapsidation (3).

A function of s1 and s1′ RNAs, apart from possibly encoding proteins, may be at the level of viral RNA transcription. Amplification of satellites by viral RDRP may down-regulate synthesis of viral RNAs and expression of their products. In this scenario, the satellite RNAs represent molecular parasites of the parental virus. Although speculative, it can be envisaged that continuous production of the satellite RNAs might be detrimental and perhaps even suicidal to both virus and satellites. This may be the reason for conditional amplification of the satellite RNAs only in specific environments. Thus, any signal for the overproduction of viral gene products can be halted by amplification of the satellite RNAs leading to the establishment of persistent viral infection. This parasitic nature has been demonstrated in yeast RNAs and in many plant satellite RNAs (10, 12, 18, 27). For example, overexpression of an S(S14) plus strand of a yeast strain by an inducible yeast expression vector has been shown to compete with and eliminate the M1 satellite dsRNA (10). In cucumber mosaic virus, synthesis of satellite RNAs reduced the level of virus replication and, interestingly, rectified the associated pathologic signs (12, 18). In addition, expression of satellite RNAs in transgenic plants has been shown to induce resistance to tobacco ringspot and cucumber mosaic viruses by down-regulating the expression of viral RNA (8, 9, 12). To this end, much more research is required to ascertain a possible function of these conditionally synthesized satellite RNAs in virus-infected *T. vaginalis.*

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