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Multiple Double-Stranded RNA Segments Are Associated with Virus Particles Infecting *Trichomonas vaginalis*

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Previous studies demonstrated that some isolates of the sexually transmitted protozoan *Trichomonas vaginalis* are infected with a nonsegmented, double-stranded RNA (dsRNA) virus. A reexamination of the total dsRNA extracted from several virus-harboring isolates indicated the presence of at least three dsRNAs with sizes ranging from 4.8 to 4.3 kbp. The double-stranded nature of each of the three segments was determined by hybridization experiments using riboprobes of opposite polarities obtained from cDNA generated to each of the segments. All three segments were present in agar clones originating from single organisms of *T. vaginalis* isolates, suggesting that the three segments were not the result of a mixed population of trichomonads harboring different sizes of dsRNA. The three segments were associated with CsCl-purified virus particles, as evidenced by electron microscopy, and RNase treatment of the preparation containing virus particles did not destroy the dsRNAs. Finally, the individual dsRNA segments were purified for use as probes to determine whether the three dsRNAs shared any sequence homology. Each end-labeled dsRNA segment did not cross-hybridize to any of the other two segments, a finding consistent with the hybridization of labeled cDNAs to only the segments from which they were derived. These results show that the coding capacity of the dsRNA virus may be at least three times greater than that estimated earlier and illustrates further the complexity of this virus-parasite interrelationship.

Double-stranded RNA (dsRNA) viruses have been discovered in pathogenic protozoa such as *Trichomonas vaginalis*, *Giardia lamblia*, *Leishmania guyanensis*, *Babesia bovis*, and several *Eimeria* spp. (15, 21). All of these protozoan viruses have been reported to have a nonsegmented genome with sizes ranging from 5.0 to 7.0 kbp (21). Virus particles having single-stranded RNA genomes with different sedimentation properties have been purified from infected leishmaniae (23, 24). A genome-length single-stranded RNA intermediate has also been detected in the total nucleic acid of virus-harboring *G. lamblia* (10). The *Giardia* and *Eimeria stiedae* viral genomes have the same electrophoretic mobility and cross-hybridize (17), suggesting a close relationship at the nucleic acid level. The *T. vaginalis* dsRNA viral genome does not share significant sequence homology with these other parasite viruses, as judged from the hybridization conditions reported (17). The effect of these viruses on the host-parasite interrelationship remains unknown, however, and requires further investigation in order to delineate contributions, if any, by these viruses to the virulence properties and pathogenesis of the parasites.

Interestingly, the replication of *T. vaginalis* dsRNA virus has been correlated with the surface expression and phenotypic variation of a highly immunogenic protein (1, 22). Elimination of detectable dsRNA from infected parasites by extended in vitro passage resulted in the loss of phenotypic variation, inability to surface express the immunogen (22), and correspondingly lower levels of cytoplasmic expression of the immunogen (3). In vivo, trichomonads harboring the virus were found to exist as a heterogeneous population of surface-expressing and non-surface-expressing trichomonads with respect to the immunogen (1), and without exception, only the virus-infected organisms switched between surface-plus and surface-minus phenotypes (1, 2, 22). When grown under continuous flow culture conditions (11), virus-harboring trichomonads have also been found to be pleomorphic, in contrast to homogeneously ellipsoid morphologic forms of isolates without detectable virus and of trichomonads which have lost the virus (10a). The inability to infect virus-minus trichomonads has been a major impediment to understanding the biological properties of the virus, the virus-parasite relationship, and the influence of the virus, if any, on expression of parasite- and/or virus-encoded virulence factors, apart from the phenotypic variation property (2).

Since the initial discovery and reports of this virus in *T. vaginalis* (19, 20), major studies describing any characterization of the dsRNA genome or the replication cycle of the virus have not appeared in the literature. Although the virus was initially described as having a nonsegmented 5.5-kbp dsRNA genome (19), further investigations by us suggested the possibility of several dsRNA segments in virus-harboring *T. vaginalis* isolates, something requiring experimental verification. The objectives of our studies, therefore, were to determine the exact number of dsRNA segments in virally infected *T. vaginalis* isolates and to examine any relationship(s) among the multiple dsRNAs segments.

This report presents evidence that all of the virus-harboring trichomonads contained at least three unique dsRNA segments with sizes ranging from 4.8 to 4.3 kbp. The three segments were found to be present in agar clones of infected parasites of several isolates and, importantly, were also found associated with purified virus particles. The results of these analysis suggest a segmented nature of the *T. vaginalis* dsRNA virus or possibly infection of this protozoan by several different viruses.

MATERIALS AND METHODS

Cultures. All isolates of *T. vaginalis* were grown in Diamond's medium supplemented with 10% horse serum (9).

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Colonies from single trichomonads of isolate T068-II were obtained by agar cloning as described previously (2). Multiple passage of T068-II and routine examination for dsRNA revealed the loss of the virus, as previously reported to occur (22), and the virus-minus progeny trichomonads were designated T068-N. These virus-negative T068-N organisms were always included throughout the studies as a negative control. Parasites were harvested at the late-logarithmic or stationary phase of growth (16) and washed in phosphate-buffered saline. These washed organisms were either used immediately for RNA and virus purification or stored at −70°C for later use.

Extraction and analysis of dsRNA. The dsRNA was extracted from total nucleic acid by the method of Chirgwin et al. (6). Briefly, 10^9 trichomonads were lysed in lysis buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate [pH 7.0], 0.1 M β-mercaptoethanol, 0.5% *N*-laurylsarcosine). The lysate was cleared by low-speed centrifugation at 10,000 × *g* before being loaded onto a 5.7 M CsCl gradient. After centrifugation at 100,000 × *g* for 24 h, the fraction containing the dsRNA was diluted in lysis buffer, and the cycle was repeated. The fraction with dsRNA was then removed, dialyzed in diethylpyrocarbonate-treated distilled water, and precipitated with 3 volumes of ethanol.

Agarose gel electrophoresis. Electrophoresis of dsRNA was performed in 1% agarose gels in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA [pH 8.0]) at a constant voltage of 40 V for 24 h (12). Electrophoresis for routine examination of dsRNA was performed at 80 V for only 2 h. Following separation, the gel was stained in TBE buffer containing 0.5 μg of ethidium bromide per ml. For cross-hybridization studies, individual RNA segments were optimally separated in 1% low-melting-point agarose, using a Max Submarine Gel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Individual segments were excised and treated with agarase enzyme (New England Biolabs, Inc., Beverly, Mass.) as instructed by the manufacturer, and the nucleic acids were precipitated with 3 volumes of ethanol.

Virus particle purification. Approximately 4×10^9 trichomonads were suspended in TNM buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$) and lysed by sonication until more than 90% of the cells were lysed (23). The lysate was clarified by centrifugation at least 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. Twenty-four fractions of 0.5 ml each were collected from the bottom of each tube and extensively dialyzed in TNM buffer. The fraction with the lightest density was designated fraction 1.

One milliliter of each fraction was then treated with proteinase K (50 μg/ml) and 1% sodium dodecyl sulfate (SDS) at 65°C for 30 min and phenol-chloroform extracted. The RNA was precipitated with 3 volumes of ethanol and separated in a 1% agarose gel as described above. Fractions containing the viral RNA were further examined for the presence of virus particles by negative staining and electron microscopy as described previously (19).

Nuclease digestion. Approximately 0.5-μg samples of purified viral RNAs were treated separately with pancreatic RNase A (100 μg/ml; Boehringer Mannheim Corp., Indianapolis, Ind.) and RNase T_1 (10 U/μg; Boehringer Mannheim) in low (50 mM) and high (500 mM) NaCl concentrations or with RQ1 DNase (10 U/μg; Promega Corp., Madison, Wis.). Each reaction mixture was incubated at 37°C for 30 min. Following phenol-chloroform extraction and ethanol precipitation, the products were analyzed in a 1% agarose gel. To determine whether the dsRNAs were possibly protected within viral capsids, the cytoplasmic fraction of lysed trichomonads was treated with RNase A (100 μg/ml) or with proteinase K (50 μg/ml) followed by RNase A. The preparations were then extracted with phenol-chloroform and precipitated with ethanol before agarose gel electrophoresis of the nucleic acids.

Generation of viral cDNA clones. Agarose gel-purified dsRNA, obtained from isolate T068-II, was denatured in 90% dimethyl sulfoxide and incubated at 65°C for 20 min in the presence of a hexamer random primer (5). First-strand cDNA synthesis was performed by using Rous-associated virus reverse transcriptase (Amersham, Arlington Heights, Ill.), and second-strand synthesis was done by the RNase H method (Amersham). The cDNA products were blunt ended by T4 polymerase followed by addition of a poly(C) tail, using terminal deoxynucleotidyltransferase (Boehringer Mannheim) (5). The C-tailed fragments were cloned into G-tailed pBR322 (GIBCO BRL, Life Technologies, Inc., Gaithersburg, Md.). Recombinants were selected on tetracycline and confirmed by the inability to grow on ampicillin plates. Three recombinants, pV12 (~1,200 bp), pV15 (~1,550 bp), and pV1 (~600 bp), which specifically and exclusively hybridized to the small (S), medium (M), and large (L) dsRNA segments, respectively, (see Fig. 2), were selected. The generated cDNAs did not hybridize to any RNA or DNA components extracted from T068-N, a virus-minus progeny of T068-II. These cDNAs were further subcloned into the transcription vector pGEM3Zf(−) (Promega) containing the SP6 and T7 promoters for generating high-specific-activity, strand-specific riboprobes.

Hybridization analysis. Approximately 100 ng of each RNA segment was electrophoresed into a nondenaturing 1% agarose gel and blotted onto Zeta probe nylon membranes (Bio-Rad Laboratories, Inc., Richmond, Calif.) as instructed by the manufacturer. Hybridization was performed overnight in the presence of 50% formamide, 100 mM Na_2PO_4 (pH 7.0), 125 mM NaCl, and 7% SDS at 42°C, using end-labeled total viral RNA or labeled individual segments. These probes were generated by partial alkaline hydrolysis (50 mM Na_2CO_3 [pH 11.0] for 10 min at 65°C) followed by incubation with T4 kinase (Boehringer Mannheim) in the presence of [γ-^{32}P]ATP at 37°C for 45 min (20). Filters were washed sequentially for 30 min each time in 2× SSC (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate [pH 7.0])–0.1% SDS, 0.5× SSC–0.1% SDS, and 0.1× SSC–0.1% SDS at 65°C (12) and exposed to X-ray film (Kodak XRP-5).

To determine the double-stranded nature of the individual RNA segments, 5 μg of total RNA from virus-harboring isolate T068-II or its virus-minus progeny T068-N, obtained by the method of Chomczynski and Sacchi (7), was electrophoresed in 1% agarose gels. Both undenatured and denatured dsRNAs, obtained by boiling in the presence of 4 M urea and 20 mM EDTA for 3 min, were loaded side by side (23). Blotting onto nylon membranes, hybridization, and washings were performed as described above. Probes of opposite polarity for each segment-specific cDNA were generated by in vitro transcription from T7 or SP6 promoters in the presence of [α-^{32}P]CTP as recommended by the manufacturer (Promega).

RESULTS

Visualization of three dsRNA segments in nucleic acid preparations of virus-infected *T. vaginalis*. Consistent with previous reports (19, 20, 22), we demonstrated by electron microscopy (Fig. 1A) and standard agarose gel electrophoretic

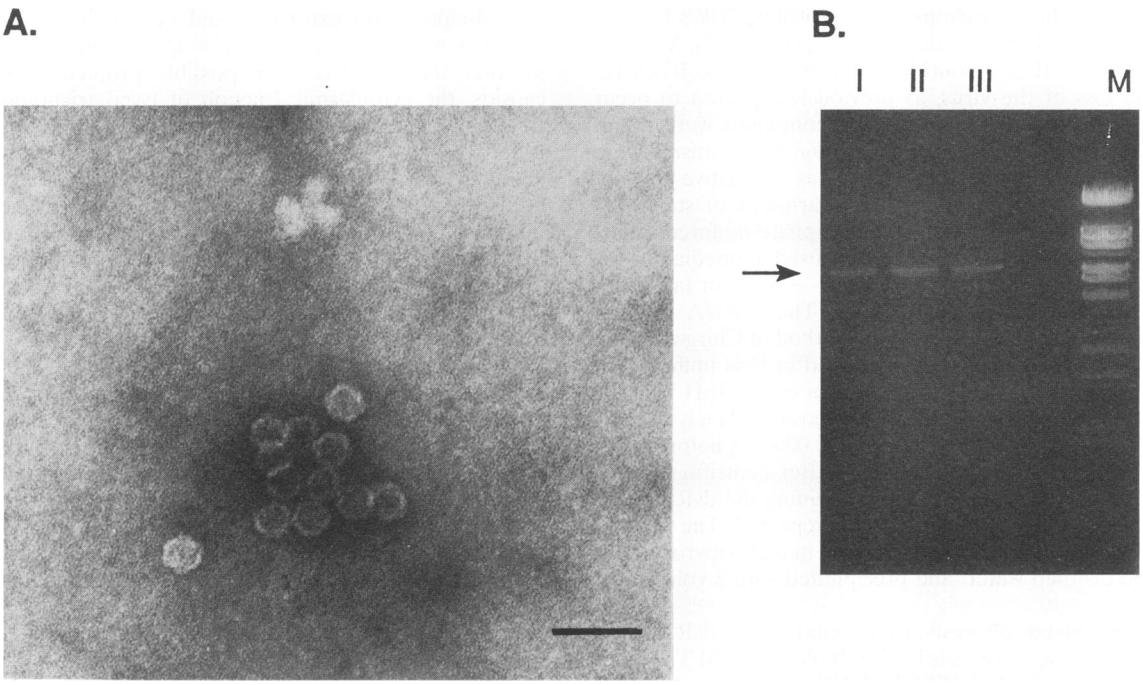

FIG. 1. Electron micrograph (A) and dsRNA genome (B) of *T. vaginalis* virus particles. (A) A sample from fraction 16 (ρ = 1.3754 g/ml) of a CsCl isopycnic gradient was directly applied to Formvar-carbon-coated grid and negative stained with uranyl acetate. Grids were examined with a JEOL-100CX electron microscope. The bar represents 100 nm. (B) CsCl fractions containing virus particles were dialyzed against TNM buffer, treated with proteinase K (50 μg/ml), and extracted with phenol-chloroform. Viral genomes were precipitated with ethanol, separated in a 1% agarose gel, and stained with ethidium bromide. Lanes I, II, and III represent viral RNA extracted from fractions 15, 16, and 17, respectively, of the CsCl gradient. The arrow indicates the position of migration of 5.5-kb dsRNA, as previously shown (19–22). Lane M contains *Pst*I-digested lambda DNA size markers.

analysis of extracted dsRNA (Fig. 1B) that some isolates of *T. vaginalis* were infected with an isometric virus containing a nonsegmented ~5.5-kbp dsRNA genome. However, close examination occasionally revealed the possibility of multiple bands in the region of the reported dsRNA viral genome. We therefore wanted to test whether the genome of the dsRNA virus of trichomonads was, in fact, segmented and contained multiple dsRNA species.

Electrophoresis of RNA extracted from several *T. vaginalis* isolates was then performed in larger agarose gels with extended separation time and low voltage. Under these improved analytical conditions, the apparent single species of dsRNA was resolved into three segments of RNA, with sizes ranging from 4.8 to 4.3 kbp (Fig. 2). It is especially noteworthy that the three segments were detected in trichomonads of the ATCC 30001 isolate; this isolate was used in earlier studies in which the original virus was purified and described (19, 20). A single-cell clone of a representative isolate (T068-IIc) also contained all dsRNA species, as did other clones of virus-harboring trichomonads (data not shown). Furthermore, trichomonal isolates that are known to be without any detectable virus, as well as T068-N, the virus-minus progeny derived from the virus-plus parental isolate T068-II (Materials and Methods), did not have any of the RNA segments (data not shown). These data show that virally infected *T. vaginalis* isolates contain at least three dsRNA segments.

The RNA nature of these segments was confirmed by nuclease treatment. All segments were found to be readily degraded by pancreatic RNase A and resistant to degradation by DNase I (data not shown), consistent with an RNA composition. These RNAs resist degradation by RNase T_1 under high salt concentrations, which permits sensitivity to pancreatic RNase A. Because of this finding, it became important to confirm the double-stranded nature of these RNA segments by Northern (RNA) blot hybridization using probes of opposite polarities, as has been done for dsRNA of *Leishmania* virus (23). Riboprobes, generated from different strands of the cDNA clones of each segment, detected a predominant single band in the nondenatured total RNA (Fig. 3, lanes 1). This was the case for each of the S, M, and L dsRNA segments. Furthermore, these riboprobes hybridized to the expected size for the single-stranded forms obtained by denaturation (lane 2) of the S, M, and L dsRNA segments. Additional subgenomic RNAs complementary to M and L segments were also detected in the total RNA of infected cells. Probes did not react with the RNA obtained from T068-N virus-minus organisms. These experiments confirmed that the RNAs were double stranded.

The three RNA segments are associated with virus particles. We next examined whether the three RNA segments were associated with virus particles. Lysates of infected cells were treated extensively with RNase A before extraction of dsRNA and examined by standard agarose gel analysis. As shown in Fig. 4 (lane 2), the dsRNAs (arrow) were resistant to RNase digestion, as evidenced by levels comparable to those seen for untreated control samples (lane 1). The RNAs were degraded if the lysate was first treated with proteinase K followed by RNase A treatment (lane 3). These data are consistent with the notion that dsRNA is protected by proteinaceous molecules.

Virus particles were next purified by CsCl density gradients to examine the association of dsRNA with distinct particles. All three segments of viral dsRNA were found in fractions 13

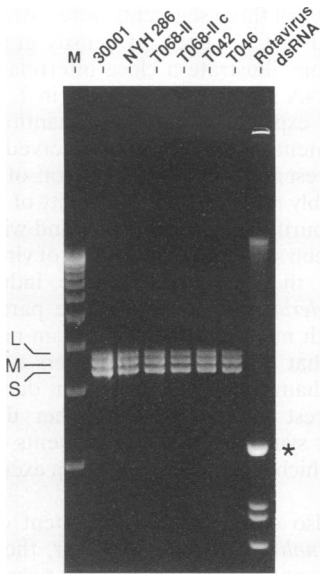

FIG. 2. Viral RNA patterns from several infected *T. vaginalis* isolates. The RNA was obtained from total nucleic acid by guanidinium lysis of cells and CsCl isopycnic gradient centrifugation. Approximately 0.5 μg of RNA, obtained from stationary-phase parasites of different isolates, was applied to a 1% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and destained in diethylpyrocarbonate-treated water. Three segments, designated L, M, and S, were detected in all of these isolates and numerous other isolates examined. Lane M represents 1-kbp dsDNA markers (GIBCO BRL). The three segments migrated between 5 and 4 kbp. Isolates ATCC 30001 and NYH 286 have been extensively studied (2, 3, 19, 20, 22). T0686-II, T042, and T046 are fresh clinical isolates. T068-IIc is representative of a single-cell, agar-cloned population derived from isolate T068-II. Rotavirus RNA was electrophoresed as a control; the slowest-migrating dsRNA segment (asterisk) is approximately 3,300 bp.

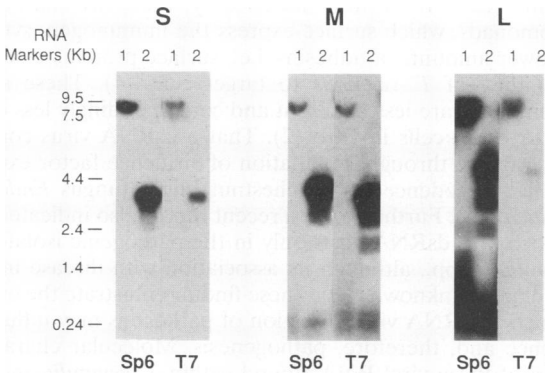

FIG. 3. Northern blot hybridization of RNA extracted from infected isolate T068-II. Total RNA was obtained as described in Materials and Methods. Approximately 5 μg of total nondenatured RNA (lane 1) was electrophoresed under normal conditions, or the RNA was denatured by boiling in the presence of 4 M urea–20 mM EDTA prior to loading for electrophoresis (lane 2). After blotting, different membranes were hybridized with riboprobes generated from either the SP6 or T7 promoter of the transcription construct specific for each of the S and M segments. The migration positions of single-stranded RNA markers (GIBCO BRL) are shown on the left.

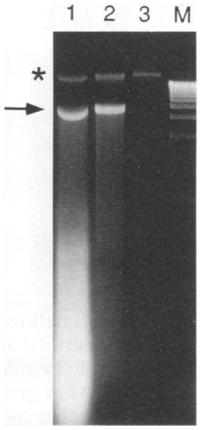

FIG. 4. Evidence that the dsRNAs are protected by proteins. Approximately 3×10^6 trichomonads were lysed by sonication, and the cytoplasmic fraction was untreated (lane 1), treated with RNase A (100 μg/ml) (lane 2), or treated first with proteinase K (50 μg/ml) and then with RNase A (100 mg/ml) (lane 3). Total nucleic acid was purified by phenol-chloroform extraction followed by standard agarose gel electrophoresis. DNA markers (lane M) are as in the legend to Fig. 2. The arrow shows the migration of the dsRNAs. The asterisk indicates the presence of contaminating cellular DNA.

to 18, with the highest concentration of these dsRNA segments found in particles banding in CsCl fraction 16 (Fig. 5). Fraction 16 had a density of 1.4 g/ml and contained isometric virus particles with a diameter of 33 nm indistinguishable from those seen in Fig. 1A. These data reaffirmed that the three RNA segments were associated with the purified virus particles.

The three segments represent unique dsRNAs. Any relationship at the nucleic acid level between the three viral dsRNA segments was analyzed by cross-hybridization experiments. Northern blot analysis using the end-labeled L segment as a

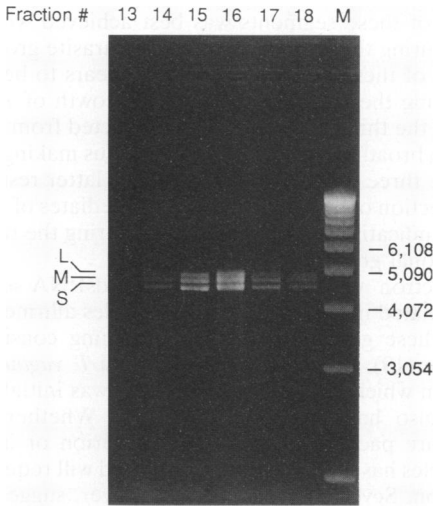

FIG. 5. The three dsRNA segments are found in virus particles purified by CsCl density gradient ultracentrifugation. The fractions were examined for the presence of viral RNA by proteinase K-SDS treatment and phenol-chloroform extraction followed by agarose gel electrophoresis as described in the legend to Fig. 2. Only fractions containing detectable amounts of viral RNA are shown. Fraction 16 had a buoyant density of 1.4 g/ml. DNA size markers are shown in lane M. Sizes are indicated in bases.

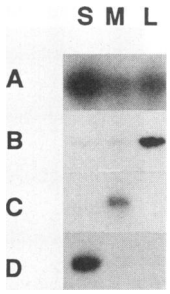

FIG. 6. Evidence for the unique nature of the three dsRNA segments. Individual segments were purified as described in Materials and Methods and electrophoresed side by side in agarose gels before blotting onto a nylon membrane. After prehybridization, separate strips were hybridized with each individual end-labeled segment (rows B, C, and D) or with all segments combined as probes (row A).

probe revealed no visible cross-hybridization with the other two segments (Fig. 6, row B). The absence of any cross-hybridization was further confirmed by experiments using the M (row C) or S dsRNA segment as a probe (row D). Hybridization signals were detected only between probe and its corresponding homologous segment. These results were confirmed by using labeled cDNA probes specific to each segment. As expected, each cDNA probe hybridized exclusively to the specific segment from which it was generated (data not shown). These results indicate uniqueness for each of the three segments isolated from *T. vaginalis* isolates infected with the dsRNA virus.

DISCUSSION

During our investigation of the viral nucleic acid within *T. vaginalis* organisms, three unique segments of dsRNA were detected. The earlier report of one segment (19) can be easily explained by the difficulty in visualizing three segments under the standard conditions of detection (Fig. 1B). Importantly, the resolution of these segments was best achieved with dsRNA obtained during the stationary phase of parasite growth, when replication of these dsRNA elements appears to be complete (10a). During the logarithmic-phase of growth of *T. vaginalis* organisms, the three dsRNA species extracted from these cells appear as a broad smear in agarose gels, thus making it difficult to visualize three different segments. This latter result may be due to detection of viral replicative intermediates of many sizes which are indicative of virus replication during the rapid phase of trichomonal growth.

That electron microscopy revealed the dsRNA segments to be encapsidated in 33-nm isometric particles affirmed the viral origin of these genetic elements, something consistent with earlier data (19). Importantly, the original *T. vaginalis* ATCC 30001, from which the trichomonad virus was initially isolated (19, 20), also had the three segments. Whether all three segments are packaged within a single virion or in separate virus particles has not been determined and will require further investigation. Several observations, however, suggest that the three dsRNA segments are associated with one virus. In studies of over 100 different clinical isolates, all virus-harboring trichomonads had the three segments described herein (Fig. 2), and parasite clones with fewer than three dsRNA segment were never detected. Equally noteworthy, all of the segments were present in cloned *T. vaginalis* cells. In addition, loss of the virus by some isolates through daily passage over prolonged periods (22) resulted in the absence of all the three dsRNA segments. Finally, all three segments were extracted from virus particles purified in CsCl buoyant density gradients (Fig. 5). These observations illustrate a close interrelationship among the distinct dsRNA segments replicating in *T. vaginalis.*

On repeated experiments, minor quantitative variations among the segments were, in fact, observed. Whether this observation represents selective replication of individual segments or is possibly related to the sensitivity of the segments to RNases during purification is unknown and will require clarification. It has been shown that formation of virus-like particles which resemble the reoviruses can be induced in a soil protozoan, *Naegleria gruberi*, when these parasites are cultivated in a growth medium containing gram-negative bacteria (18). Knowing that *T. vaginalis* encounters a highly complex and constantly changing host environment during infection, it will be of interest to determine whether different growth conditions affect synthesis of these segments or induction of other dsRNAs which cannot be detected in axenic and/or batch cultures.

Our results also show that each segment of the virus(es) infecting *T. vaginalis* is unique. However, the segments may share some sequence similarities, such as polymerase recognition sites and encapsidation signals, which cannot be detected by hybridization studies. That these segments do not share any sequence homology with host DNA (data not shown) is consistent with the initial report (19). Earlier studies indicated that the dsRNA genome of the *T. vaginalis* virus does not hybridize to dsRNA viruses of other protozoa (17). However, these earlier reports await a reexamination, since the exact composition of viral nucleic acid derived from the trichomonads used in those experiments is now unclear.

The relationship between the presence of a dsRNA virus and phenotypic variation in *T. vaginalis* shows that this virus-parasite interaction may have consequences for the host during infection. For example, virus-harboring trichomonads synthesize an increased amount of a highly immunogenic, phenotypically varying outer membrane protein compared with uninfected *T. vaginalis* isolates. This observation is corroborated by finding increased levels of mRNA synthesis for the immunogen (8, 10a). This upregulation of major immunogen expression, along with its surface localization (1–3), appears to be aborted when the dsRNA virus is lost through in vitro cultivation of the parasite (22). It is also noteworthy that virus-harboring trichomonads, which surface express the immunogen, synthesize lower amounts of adhesins, i.e., surface proteins essential for binding of *T. vaginalis* to target cells (4). These same trichomonads are less adherent and correspondingly less cytotoxic to HeLa cells in vitro (2). That a dsRNA virus confers hypovirulence through modulation of virulence factor expression has precedence in the chestnut blight fungus *Endothia parasitica* (14). Furthermore, a recent report also indicated the presence of a dsRNA virus only in the pathogenic isolates of *Phytomonas* spp., although its association with disease induction remains unknown (13). These findings illustrate the extent to which a dsRNA virus infection of pathogens may influence virulence and, therefore, pathogenesis. Molecular characterization of these viral RNAs found within *T. vaginalis* and the establishment of conditions which will allow for infection of isolates without detectable virus and/or viral dsRNAs may eventually elucidate the relationship between the virus and virulence properties of this sexually transmitted protozoan.

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