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Trichomonas vaginalis with a Double-Stranded RNA Virus Has Upregulated Levels of Phenotypically Variable Immunogen mRNA

ALI KHOSHNAN AND J. F. ALDERETE*

Department of Microbiology, The University of Texas Health Science Center, San Antonio, Texas 78284-7758

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Some isolates of *Trichomonas vaginalis* carry a double-stranded RNA virus (TVV) and undergo phenotypic variation between surface expression and cytoplasmic expression of a prominent immunogen (P270). Only trichomonads with TVV express P270 on the surface. Northern (RNA) blots using a specific cDNA encoding the repeat element of the phenotypically varying P270 immunogen as a probe showed accumulation of P270 transcript only in isolates with TVV (V^+) in contrast to isolates without the virus (V^-). To test further the influence of virus infection on P270 mRNA expression, V^- progeny, derived from the parental V^+ isolates, were tested. Trichomonads of V^- progeny, like the fresh clinical V^- isolates, also showed no accumulation of P270 mRNA. By immunoblotting with a monoclonal antibody to the key repeated epitope of P270, it was found that V^- organisms had quantitatively less immunoreactive protein than the parental V^+ isolates. Although V^+ and V^- isolates contained proteins immunoreactive with the monoclonal antibody to P270, it was necessary to test for the presence of the P270 gene among all the isolates. As expected, Southern blots demonstrated that V^+ and V^- trichomonads possessed the gene encoding P270. These data suggest that the double-stranded RNA virus of *T. vaginalis* is involved in the regulation of P270 mRNA accumulation.

Some isolates of *Trichomonas vaginalis*, a sexually transmitted protozoan, are persistently infected with a double-stranded RNA (dsRNA) virus called TVV (18, 21). The virus is not detectable in the supernatant of infected cultures. Attempts to infect trichomonads without TVV (V^-) with purified viral particles have had limited success (12, 20, 21). Recently, it was discovered that TVV is multisegmented, having at least three dsRNA segments ranging in size from 4.3 to 4.8 kb. These segments copurified with isometric viral particles of 33 nm in diameter (11). Cross-hybridization studies revealed no homology among the segments, showing an increase in the coding capacity of TVV (11) from that estimated earlier (20). Characterization of the mechanism of viral replication and its encoded products has not been reported.

Two types of *T. vaginalis* isolates have been described with respect to surface expression and phenotypic variation of a major immunogen called P270 (1, 2, 4, 6). Type I isolates were homogeneous populations of parasites that synthesized P270 but were unable to express the immunogen on the surface. Type II isolates, on the other hand, comprised a heterogeneous population of trichomonads in which some parasites had P270 on the surface (2, 4, 6). Each subpopulation of type II organisms, however, underwent phenotypic variation between surface and cytoplasmic expression of P270 (4). Only type II isolates were found to harbor TVV (V^+) (22), and interestingly, loss of TVV from type II parasites resulted in a transition of trichomonads toward type I (V^-) (4, 22). Therefore, the relationship between the presence of TVV and phenotypic variation indicated that the virus influenced surface expression of P270.

To establish whether the presence of TVV affected overall expression of P270, we first decided to use a recently generated cDNA, which encodes the repeat sequence of P270 (9), as a probe to evaluate mRNA levels among V^- and V^+ isolates. Figure 1 presents representative results of the numerous isolates examined. As can be seen, only blots with total RNA from V^+ isolates, shown in lane A for a representative V^+ isolate (TO68-II), had a detectable band for P270 mRNA. An ~9.0-kb mRNA for P270 has been previously described (9). Under the same conditions, no bands were evident for any of the V^- isolates (SA92-80, TO76, TO77, and TO80) when total RNA was examined. As controls, the duplicate gels of ethidium bromide (EtBr)-stained total RNA are given to confirm that equivalent amounts of RNA were added to each well. These data suggest a possible relationship between accumulation and, therefore, detection of mRNA for P270 and the presence of the dsRNA virus in trichomonads.

To extend these initial observations, several V^+ *T. vaginalis* isolates were cloned in soft agar (4) and grown for extended periods in order to obtain cloned V^- progeny, as described before (22). Criteria used to assign whether trichomonads either do or do not have TVV are shown in Table 1. First, no detectable viral particles could be purified by CsCl (11, 20) from V^- organisms, as was readily done with V^+ parasites under identical experimental conditions. Second, no dsRNA bands were visible by EtBr staining of agarose gels after total nucleic acid electrophoresis of V^- progeny organisms (11, 20, 22). This method has been routinely used to show the presence of TVV in trichomonads (20). Finally, the viral genomes were not detected by Northern blots using riboprobes of recently generated cDNAs to each of the dsRNA segments in any of the V^- isolates or progeny (11).

As shown in Fig. 2A, Northern blots revealed bands only in V^+ parasites. This RNA species was not detectable in any of the samples of V^- progeny trichomonads when equal amounts of total RNA were examined simultaneously under the same experimental conditions. Furthermore, even the addition of greater amounts of total RNA from V^- organisms in Northern blots failed to give a signal (data not shown). These data were

* Corresponding author. Mailing address: Department of Microbiology, The University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78284-7758. Phone: (210) 567-3940. Fax: (210) 567-6612.

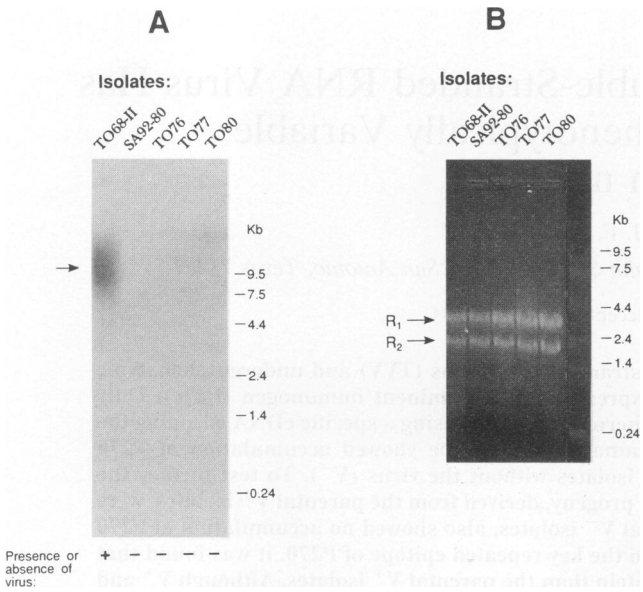

FIG. 1. Detection of P270 mRNA in V^+ and V^- isolate trichomonads. (A) Total RNA was isolated from fresh isolates of *T. vaginalis* by the methods of Chomczynski and Sacchi (7). Approximately 15 μg of RNA from each isolate was resolved by denaturing formaldehyde agarose gel electrophoresis (15), followed by transfer to a Zeta probe membrane in 50 mM NaOH (Bio-Rad Laboratories, Richmond, Calif.). Membranes were prehybridized for 2 h at 42°C in a solution having 50% formamide, 100 mM Na_2PO_4 (pH 7.0), 125 mM NaCl, 7% sodium dodecyl sulfate (SDS), 100 μg of salmon sperm DNA per ml, and 20 μg of yeast RNA per ml. Hybridization was carried out overnight at 42°C in the same solution containing 10^7 cpm of nick-translated [α-^{32}P]dCTP-labelled P270 cDNA probe. Membranes were then washed sequentially for 3 min 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 65°C and exposed to Kodak XAR-5 X-ray film. The ~9.0-kb mRNA is shown (arrow) in TO68-II, a representative V^+ isolate. Fresh V^- isolates, SA92-80, TO76, and TO77, and a representative long-term-grown isolate, TO80, were examined similarly. (B) EtBr staining of the gel prior to blotting as a control to show loading of equivalent amounts of total RNA. Single-stranded RNAs (GIBCO-BRL, Life Technologies, Inc.) used for the size estimations are shown on the right.

in agreement with those of Fig. 1 in that the loss of TVV resulted in a reduction of P270 mRNA synthesis or a lack of accumulation possibly due to increased mRNA turnover. The size polymorphism of the P270 mRNA was not surprising and was consistent with the known heterogeneity of P270 molecular weights (6).

Since trichomonads of fresh clinical V^- isolates synthesize low levels of P270 proteins (5), it was essential that the V^- progeny with no detectable P270 mRNA also be examined for synthesis of the protein immunogen. Figure 3A shows that V^- organisms (lanes N) still synthesized decreased amounts of P270 compared with those of V^+ organisms (lanes P). This was evidenced by immunoblot analysis of the total lysate with the monoclonal antibody, C20A3, that recognizes the single repeated epitope of P270 (5, 9). Degradation (noted by the asterisk), visualized by the appearance of multiple bands and smearing in immunoblots of the native P270 (indicated by the arrow), was described previously (5) and shown to be due to autodegradation of the protein by trichomonad proteinases (5, 8, 16) released during processing. The complex total protein patterns of Coomassie brilliant blue-stained gels of identical

TABLE 1. Presence and absence of TVV in *T. vaginalis* isolates

Isolate[a]	Detection of viral:	
	Particles[b]	Genome[c]
Parental V^+		
AL8P	+	+
AL10P	+	+
347P	+	+
8111P	+	+
Progeny V^-		
AL8N	−	−
AL10N	−	−
347N	−	−
8111N	−	−

[a] Parental isolates (P) with V^+ trichomonads were subjected to in vitro cultivation with daily passage in a complex medium (6, 10). Progeny organisms without the virus (N) were derived from numerous parental, agar-cloned trichomonads (4) after extended in vitro passage as described before (10, 22). During daily passage of cultures, trichomonads were examined for presence or absence of the virus as described below.

[b] Established procedures were employed to observe the banding of viral particles (11). In this case, viral particles were purified from the cytoplasmic fraction of 4×10^9 cells by CsCl density gradients. In parental V^+ isolates, viral particles banded at a density of 1.4 g/ml and were observed by electron microscopy, as had been reported before (11). The existence of viral particles at this density was confirmed by detection of viral RNA. Following proteinase K treatment (50 μg/ml), phenol-chloroform extraction, and ethanol precipitation of CsCl fractions, viral genomes were visualized by standard agarose gel electrophoresis and EtBr staining (11).

[c] Two methods were used to detect the viral dsRNAs (11) in trichomonads. Routine examination of the test tube cultures for the presence of viral dsRNA genomes was by agarose gel electrophoresis and EtBr staining of the total nucleic acid, as presented above (11). In this procedure, dsRNAs migrated at ~5.0 kb (11). This band was always absent from total nucleic acid preparations derived from the V^- progeny. Absence of viral genomes was also confirmed by Northern blots using riboprobes generated to the three dsRNA segments of the viral genome (11). In this case, total RNA was examined as described in the legend to Fig. 1. Hybridization was performed in the presence of [α-^{32}P]CTP-labelled riboprobes generated from a cDNA cloned in pGEM3ZF(−) carrying the SP6 and T7 promoters (Promega Corp., Madison, Wis.). No hybridization signals were ever detected in Northern blots using total RNA from the V^- progeny.

cell numbers (Fig. 3B) indicate that equivalent amounts of protein were loaded for the V^+ parental and V^- progeny samples. These results reaffirmed earlier observations with other isolates (2, 4–6) that P270 protein was expressed in larger amounts in V^+ (type II) parasites and that loss of the virus resulted in concomitant decreased synthesis of C20A3-immunoreactive proteins (5).

Although all isolates made proteins immunoreactive to the monoclonal antibody C20A3 (2, 4, 6), it was equally important to establish that the P270 gene was present in all V^+ and V^- parasites. This was done by Southern blot analysis using as a probe the 330-bp *Hin*dIII cDNA that encodes for the epitope that is tandemly repeated 12 times in the gene (9). Fig. 2B provides evidence that the gene was indeed present in all V^+ isolates and V^- progeny trichomonads. The same intensity in hybridization was detected in all lanes of DNA from either V^+ or V^- parasites.

Finally, consistent with earlier work (4, 22), flow cytofluorometry revealed that all V^+ parental isolates had organisms which expressed P270 on the surface and underwent phenotypic variation (data not shown). Under the same conditions, V^- progeny remained unable to place P270 on the surface during an extended period of evaluation.

Early on, we examined the relationship between the dsRNA virus (11, 20) and phenotypic variation of the protein P270 (2, 4, 6, 9). We now present evidence that the mRNA of P270 is more abundant during TVV infection of trichomonads, and

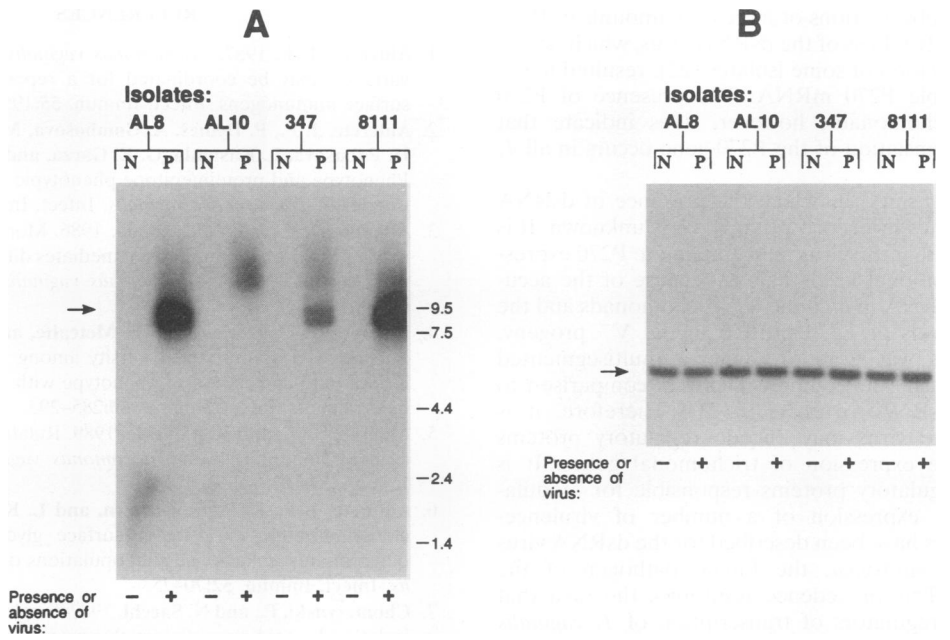

FIG. 2. Northern blot (A) and Southern blot analysis (B) of P270 mRNA and DNA, respectively, in V^+ parental trichomonads (lanes P) and V^- progeny organisms (lanes N). (A) Preparation of RNA and detection of P270 mRNA were as described in the legend of Fig. 1. The arrow shows the accumulation of P270 mRNA in the V^+ trichomonads but not in the V^- trichomonads. Single-stranded RNA markers were as described in the Fig. 1 legend. (B) DNA (10 μg) extracted from parasites was digested to completion with *Hin*dIII. After separation by electrophoresis and transfer to nylon membranes, the DNA was probed with the 330-bp cDNA as described before (9). Hybridization conditions were as described in the legend of Fig. 1. The arrow denotes the existence of the repeat fragment in all V^+ and V^- progeny.

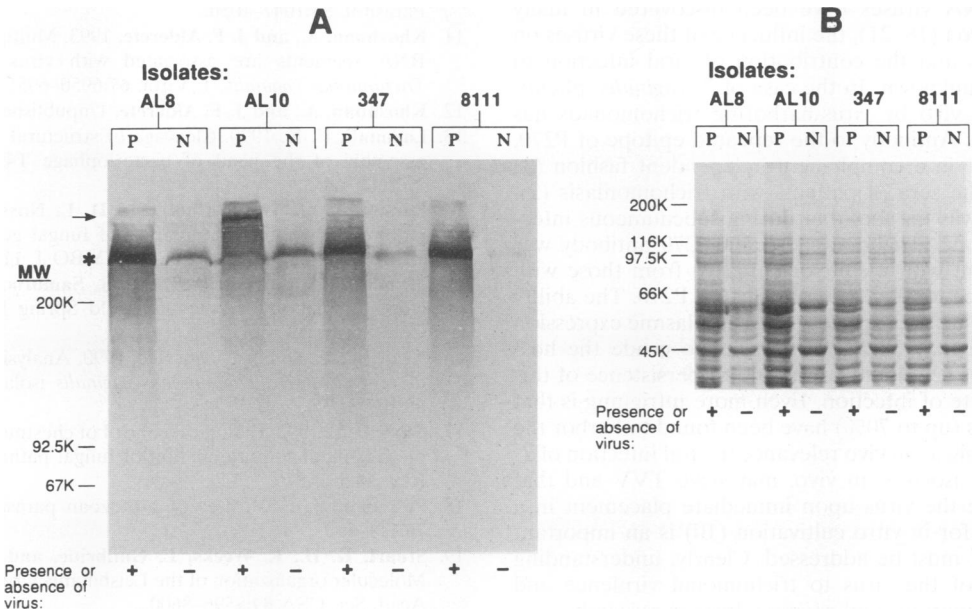

FIG. 3. Immunoblot detection of P270 (A) and Coomassie brilliant blue staining of total proteins (B) of V^+ (lanes P) and V^- (lanes N) trichomonads after SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Total cell proteins from 2×10^6 trichomonads were precipitated with 10% trichloroacetic acid at 4°C as described previously (6), solubilized in dissolving buffer (125 mM Tris-HCl [pH 8.0], 14% SDS, 20% glycerol, 5% β-mercaptoethanol), and boiled for 5 min prior to SDS-PAGE in 7.5% acrylamide gels by standard procedures (13). Following separation, proteins were blotted onto nitrocellulose by electroblotting in 20 mM Tris-HCl (pH 8.0)–150 mM glycine–20% methanol. Membranes were treated with a solution of 5% nonfat dry milk powder and reacted with the monoclonal antibody C20A3 (6). A secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Sigma) was then added, and membranes were developed according to established procedures. (B) Corresponding Coomassie brilliant blue-stained patterns of total proteins derived from equal cell numbers of V^+ and V^- samples electrophoresed simultaneously. Molecular weight (MW) markers (Bio-Rad Laboratories) are on the left (in kilodaltons). The asterisk indicates degradation, and the arrow indicates intact P270 protein.

this corresponds to observations of increased amounts of P270 in type II parasites (16). Loss of the dsRNA virus, which occurs during in vitro cultivation of some isolates (22), resulted in the absence of detectable P270 mRNA. The presence of P270 protein in V^- trichomonads, however, does indicate that transcription and translation of the P270 gene occurs in all *T. vaginalis* organisms.

The exact mechanism(s) by which the presence of dsRNA influences or regulates levels of P270 mRNA is unknown. It is tempting to suggest that the virus may upregulate P270 expression at the transcriptional level. This is because of the accumulation of P270 mRNA in only the V^+ trichomonads and the presence of low levels of P270 protein in the V^- progeny. Recently, this virus was found to have a multisegmented genome (11), increasing its coding capacity in comparison to other protozoan dsRNA viruses (19, 21). Therefore, it is conceivable that the virus may encode regulatory proteins which influence the expression of trichomonal genes. It is noteworthy that regulatory proteins responsible for modulation of phenotypic expression of a number of virulence-associated properties have been described for the dsRNA virus infecting *Endothia parasitica*, the fungal pathogen of the chestnut (14, 17). This precedence reinforces the idea that TVV may encode regulators of transcription of *T. vaginalis* genes like P270, something that will require experimental verification.

An alternative explanation for the inability to detect P270 mRNA in V^- organisms is that the turnover of mRNA is much greater in V^- than V^+ parasites. In this scenario, V^+ trichomonads may possess factors, either of host or viral origin, which inhibit mRNA degradation. This would allow for more stability and an extended half-life of mRNA, which would yield greater amounts of mRNA in the V^+ trichomonads.

Although dsRNA viruses have been discovered in many pathogenic protozoa (18, 21), the influence of these viruses on the parasite hosts and the contribution of viral infection to pathogenesis are unknown. In the case of *T. vaginalis*, phenotypic variation in vivo by virus-harboring trichomonads has been shown (1, 2). Antibody to the repeated epitope of P270, in fact, is cytolytic in a complement-independent fashion (3) and is found in the sera of patients with trichomoniasis (2). Also noteworthy was the fact that during subcutaneous infection of mice with V^+ trichomonads, anti-P270 antibody was detected coincident with a shift of parasites from those with P270 on the surface to those without surface P270. The ability to alternate from surface expression to cytoplasmic expression of an immunogen may allow the parasite to evade the host antibody responses (2, 6) and contribute to persistence of this pathogen at the site of infection. Even more intriguing is that most fresh isolates (up to 70%) have been found to harbor the virus (12), indicating an in vivo relevance to viral infection of *T. vaginalis*. That all isolates, in vivo, may have TVV and that some isolates lose the virus upon immediate placement in a complex medium for in vitro cultivation (10) is an important consideration that must be addressed. Clearly, understanding the contribution of the virus to trichomonal virulence and pathogenesis is important and requires further research.

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