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The *Trichomonas vaginalis* phenotypically varying P270 immunogen is highly conserved except for numbers of repeated elements

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The prominent and phenotypically variable immunogenic protein of *Trichomonas vaginalis*, termed P270, is present in all isolates. Most, if not all, patients make antibody to the DREGRD epitope contained in the 333 bp tandemly repeating element (TRE). The complete sequence of *p270* of a fresh clinical isolate was recently derived (Musatovova and Alderete, *Microb Pathogen* 1998; 24: 223–39). We hypothesized that the size polymorphisms of P270 were due to the varied number of TREs that comprise a large, central portion of the gene. In this study, we analysed the *p270* coding regions of ten representative isolates. It was determined also that the sequence of the TRE of different *p270* genes shared $\geq$99% identity, and individual TREs of the same *p270* gene showed them to have identical nucleotide sequences, affirming the highly-conserved nature of this element within each gene. The coding regions upstream and downstream of the central TREs were then generated by PCR amplification using specific primers. The PCR products corresponding to the 5′ and 3′-end coding, non-repeat sequences were then subjected to restriction analyses, and the regions were highly conserved for all *p270* genes. The complete sequence of two *p270* genes showed $\geq$99% identity of amino acids at the N- and C-terminal regions of *p270*, further reinforcing that the reported polymorphisms in M_r of P270 is due to the varying number of TREs and, therefore, the size of the TRE domain. In support of this hypothesis and during these analyses, one isolate, *T. vaginalis* T016, was discovered which possessed a *p270* gene with only one partial repeat unit. Importantly, and as with all other *p270* genes, transcription of this single-repeat *p270* gene in isolate T016 was confirmed. The start codon for the *p270* T016 gene was preceded by the 12 nucleotide consensus Inr promoter-like sequence (TCATTTTTAATA) and possessed a putative transmembrane domain at the carboxy terminus.

Key words: host-parasite, tandemly repeating element (TRE), *Trichomonas vaginalis*, virulence.

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Introduction

Infection with *Trichomonas vaginalis* remains unappreciated; yet, trichomonosis is the number one non-viral, sexually transmitted disease (STD) worldwide [1]. This STD has major health consequences for women, including predisposition to HIV [2–6], association with cervical cancer [7, 8], and complications during pregnancy [9–12]. The long-range goal of this research project is to understand the pathogenesis of trichomonosis. The high M_r protein, P270, with tandemly repeating elements (TREs), each of which contains the immunogenic DRE-GRD epitope recognized by the monoclonal antibody (mAb) C20A3 is important in the *T. vaginalis*-host relationship [13–20]. Our studies have shown that mouse monoclonal antibody (mAb) [13] or polyclonal Ab from sera of infected women [14] is cytolytic for trichomonads with surface P270. The parasite has the capacity to modulate production and surface expression of P270 [14–17], thereby avoiding host destruction by Ab [13]. Examination of more than one-thousand isolates from different geographic regions has revealed that 50% of isolates harbor a unique, segmented dsRNA virus, the presence of which is related to up-regulation of transcription [19] and levels of expression of P270 by at least two orders of magnitude [15, 16, 18, 19]. Importantly, the presence of this virus is required for surface expression and phenotypic variation of the P270 [21]. Thus, the virus provides a marker from which to carry out comparative studies. Further, a repertoire of coordinately-regulated, phenotypically varying proteins, of which P270 is a member, is synthesized by *T. vaginalis* [22].

Analysis of other trichomonal isolates using mAb C20A3 revealed size polymorphism among P270s [15, 19]. Further, the relatedness of the different P270s was confirmed by identical immunoblot ladder patterns following proteolysis through autodegradation [17]. Recently, the *p270* gene sequence of a fresh clinical isolate, *T. vaginalis* T068-II, was reported [20] and revealed the presence of at least eighteen 333 bp TREs [18] located within the gene. The repeated domain was flanked by 69 bp (23 amino acids) of upstream and 1188 bp (395 amino acids) of downstream non-repeat, coding regions [20]. The sequences of the repeats within this *p270* gene were identical [20].

In this paper, we present data to show the highly-conserved nature of the distinct parts of the *p270* genes of different isolates. Data strongly suggest that the size polymorphism of P270 among isolates is due to the different numbers of repeats within the P270 TRE domain. Finally, an isolate without dsRNA virus was discovered that had a *p270* gene containing only one partial repeat. The amino acid sequence of this novel *p270* gene was $\geq$99% identical to the known sequence of *p270* [20]. The data presented here suggest a role for variables other than those inherent within P270 in the regulation of surface placement of P270. The significance of our results are discussed, especially given the precedence that the number of repeat units within antigens is related to microbial virulence and pathogenesis.

Results

P270 proteins and *p270* genes among representative *T. vaginalis* isolates

It was important to show the existence of immuno-crossreactive P270s of different sizes among representative isolates. The P270s were first precipitated by mAb C20A3 from detergent extracts of the representative *T. vaginalis* virus-harboring isolates T068-II and NYH 286 (the common laboratory isolate used originally [13–18]) and virus-minus isolate T080 [13–15] followed by electrophoresis and immunoblot visualization [Fig. 1(a)]. Size polymorphisms among the immunoprecipitated P270s were evident, as before [15, 17, 19]. The multiple bands seen in stained gels on occasion were from the autodegradation of P270 by the trichomonad cysteine proteinases released during detergent solubilization of parasites [17]. In data not shown, limiting autodegradation experiments performed on trichomonal detergent extracts monitored by immunoblotting with mAb C20A3 after SDS-PAGE [17] showed identical ladder digestion patterns, indicating a relatedness of P270s at the protein-structure level [17].

Southern analysis was also performed on genomic DNA using several restriction enzymes, such as *Eco*RI, that cut outside the tandemly repeated region [20]. Blots were probed with ^{32}P-labeled cDNA encoding the TRE [18], and, not unexpectedly, large fragments were detected [Fig. 1(b)] with sizes proportional to the M_r of P270 in these isolates [Fig. 1(a)]. Further, *Hind*III that cuts once within the TRE yielded identical

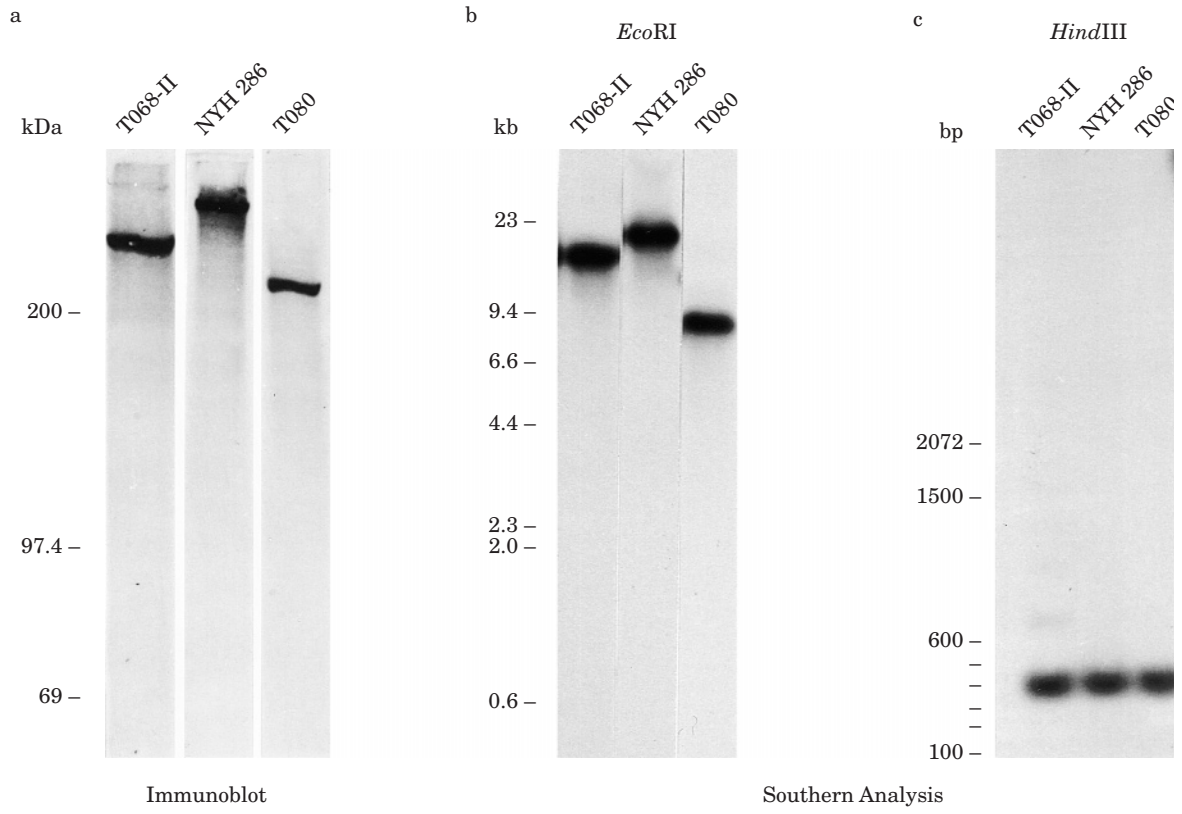

Figure 1. Size polymorphisms of P270 and characterization of the *p270* genes of representative *T. vaginalis* isolates T068-II, NYH 286, and T080. (a) Size heterogeneity of P270 was demonstrated by purifying proteins by immunoprecipitation as described in Materials and methods. After electrophoresis and blotting of proteins onto nitrocellulose, the P270 was visualized by using the mAb C20A3 as the probe [15]. Numbers and kDa on the left represent size markers in kilodaltons (k = 1000). (b) and (c) Trichomonal genomic DNA (5 μg) was purified and digested to completion with *Eco*RI and *Hind*III prior to separation on 1% agarose gels followed by transferring onto Zeta-probe membranes. Hybridization under high stringency was performed using ^{32}P-labelled cDNA representing the repeat sequence. The *Hind*III digest of λDNA (b) and the 100-bp DNA ladder (GibcoBRL) (c) were used here and in all other figures as size markers.

333 bp hybridizing fragments [Fig. 1(c)], and partial digestion experiments with *Hind*III [18] gave identical ladder patterns with equidistant hybridizing bands and accumulation of the smallest fragment over time when probed with the radiolabeled repeat (data not shown). This shows the tandemly repeating nature of a significant portion of the *p270* genes. These and other P270 proteins and genes studied similarly provide data consistent with the model for *p270* as shown in Fig. 2(a) for isolate T068-II [20].

The 3′-end non-repeat, coding region of *p270* is conserved among isolates

We wanted to know whether the non-repeat, coding sequence downstream of the TRE domain was conserved. PCR amplification of this region of the *p270* genes was performed on genomic DNA of representative trichomonal isolates using primers designed based on sequence data recently generated [20]. To avoid binding of multiple primers to a template, both primers were intentionally localized within the non-repeat, coding sequence. Importantly, as shown in Fig. 3, representative isolates without dsRNA virus (T016 and T080) and the remaining dsRNA virus-infected isolates were analysed. All isolates gave a PCR product of ~900 bp that was identical to the product for T068-II, the reference isolate [20]. The use of alternative primers within the non-repeat, coding region to yield different-sized PCR products from throughout this region also gave identical banding patterns for all isolates. Finally, restriction analyses was performed

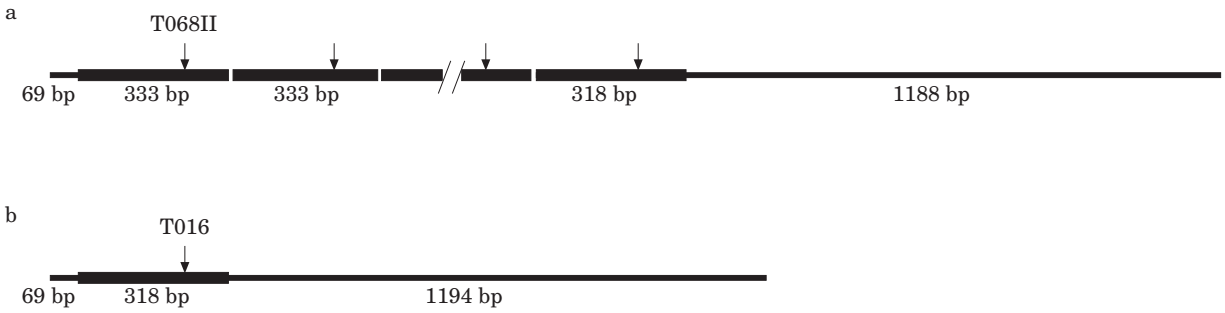

Figure 2. Model of the *p270* gene constructed from experiments with the fresh clinical *T. vaginalis* isolate T068-II [20] and other isolates (a) compared with that of isolate T016 (b). Isolate T068-II has at least eighteen 333 bp TREs defined by a *Hind*III restriction site [18, 20]. Isolate T016 has only one partial repeat element of 318 nucleotides, corresponding to the sequence of the most 3′-end repeat of isolate T068-II. Note the identical 69 nucleotide sequence of the 5′-end non-repeat, coding sequence and the minor variance at the 3′-end non-repeat, coding sequence. Greater details for both *p270* genes are provided in the text and in Fig. 7.

on the PCR products. Based on the known DNA sequence for *p270* of isolate T068-II [20], restriction sites for *Bam*HI, *Eco*RV, *Hind*III, and *Kpn*I were not found within the 3′-end non-repeat region, and enzymes *Acc*I, *Eco*RI, and *Hae*III cut once. Not unexpectedly, restriction with the enzymes yielded identical patterns for all 3′-end amplified DNA for both cutting and non-cutting enzymes (data not shown), affirming the conservation of this *p270* gene region.

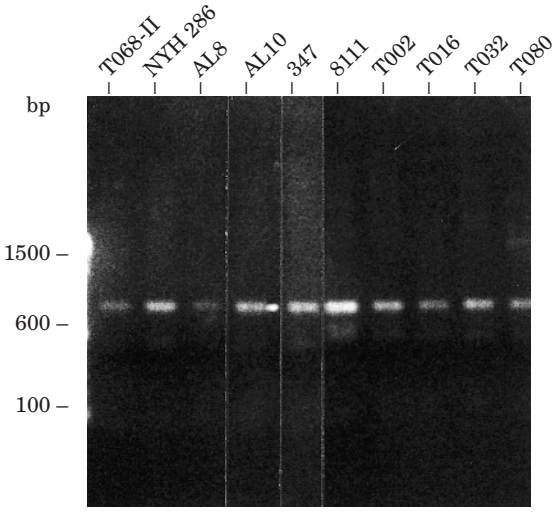

Figure 3. PCR amplification of the coding region downstream of the TRE domain. Aliquots of the PCR amplification reactions for ten *T. vaginalis* isolates indicated above each lane were electrophoresed in 1% agarose and compared to the expected product of the control PCR amplification reaction performed for T068-II genomic DNA. DNA was stained with ethidium bromide.

The 5′-end coding and upstream intergenic sequence has high identity among *p270* genes

Evaluation of the coding region upstream of the TRE domain was performed. Because the coding sequence was only 69 bp for the 5′-end of isolate T068-II *p270* [20], we had to utilize a sense primer that was localized in the adjacent intergenic sequence and that was successfully used previously for amplification of *p270* [20]. The complement of the sequence coding the DREGRD epitope, known to be present in each repeat, was used for the antisense primer. Predictably and as seen before [20], multiple binding of the antisense primer in the tandemly repeated elements resulted in the generation of multiple, equidistant higher-sized PCR products (Fig. 4). The larger DNA products were from the addition of increased numbers of 333 bp TREs. Digestion of the higher-sized products with *Hind*III resulted in the 333 bp repeat unit in addition to the intergenic and coding, non-repeat DNA band. The smallest product was of identical size for the *p270* gene of all isolates, indicating both the conservation of the coding sequence upstream of the TREs and of the adjacent upstream intergenic sequence. The conserved nature of the amplified sequences derived for all isolates was further confirmed by restriction after ligation of the PCR products into pCR®2.1.

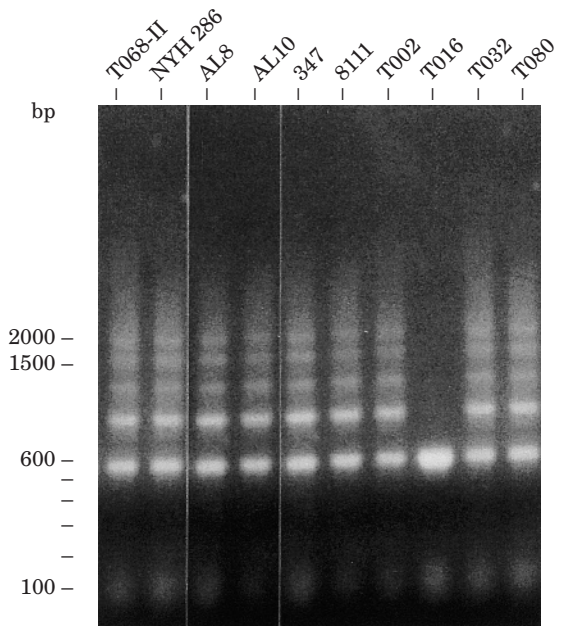

Figure 4. Amplification of the sequences at the 5′-end of the TRE domain, which includes both the 69 nucleotide non-repeat, coding sequence plus upstream intergenic sequence, as characterized before for T068-II [20]. Aliquots of PCR reactions for ten *T. vaginalis* isolates were electrophoresed in 1.2% agarose and stained with ethidium bromide. Patterns were compared with the control PCR amplification reaction performed on T068-II genomic DNA, as recently reported [20]. Note the intense single band below the 600 bp marker when the PCR amplification reaction used genomic DNA of isolate T016. Both T016 and T080 are representative of *T. vaginalis* isolates without the double-stained RNA virus [19, 21].

The *p270* of *T. vaginalis* isolate T016 has only one repeat element

Surprisingly and unexpectedly, a single product was obtained during the above PCR amplification for the *p270* gene of *T. vaginalis* isolate T016 (Fig. 4). This suggested that the epitope coding sequence was conserved only in the first repeat or that only one repeat element was contained in this gene. Southern analysis was then performed on the genomic DNA restricted with *Eco*RI using cDNA encoding the repeat as a probe. As seen in Fig. 5, the autoradiogram showed a band smaller than that seen for the *p270* DNA fragments of other isolates handled similarly and that hybridized with the repeat element, as shown earlier in Fig. 1(b). Restriction with *Hind*III that cuts once within each repeat (Fig. 2) yielded two hybridizing fragments of

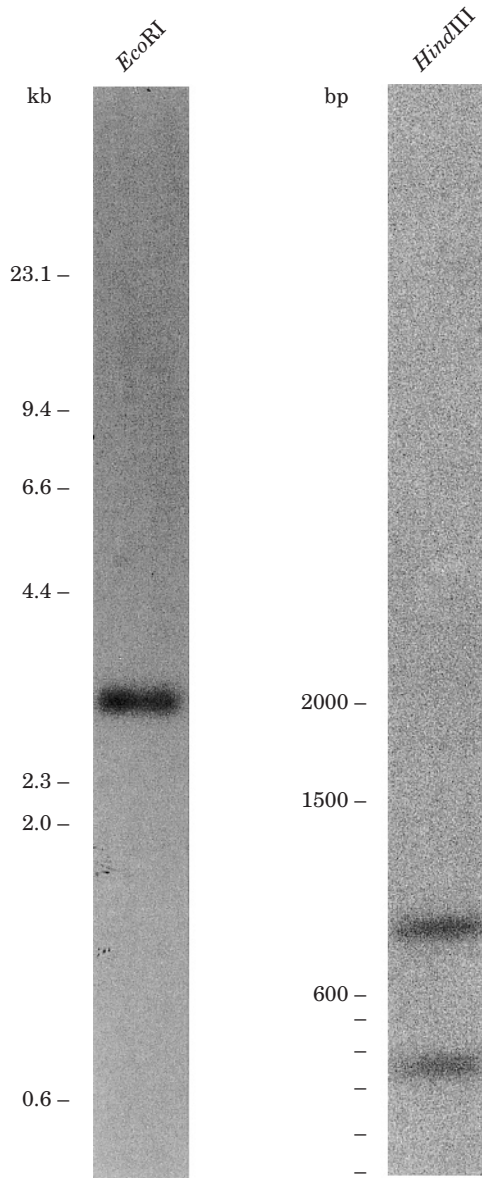

Figure 5. Southern analysis of *T. vaginalis* isolate T016. Purified genomic DNA (5 μg) was completely digested with *Eco*RI or *Hind*III. Restriction fragments were separated by electrophoresis in 0.8% and 1.2% agarose gels, respectively, for *Eco*RI- and *Hind*III-digested DNA. The digested DNA was then blotted onto Zeta-probe membranes and hybridized with nick-translated cDNA representing the TRE [18, 20].

expected sizes, further consistent with a single *Hind*III site within the repeat. These findings prompted us to perform PCR amplification in an attempt to generate the complete *p270* coding sequence of isolate T016. As can be seen in Fig. 6, using primers within the 5′- and 3′-end non-repeat, coding sequence, a PCR product of ~1600 bp was obtained, reinforcing the idea that

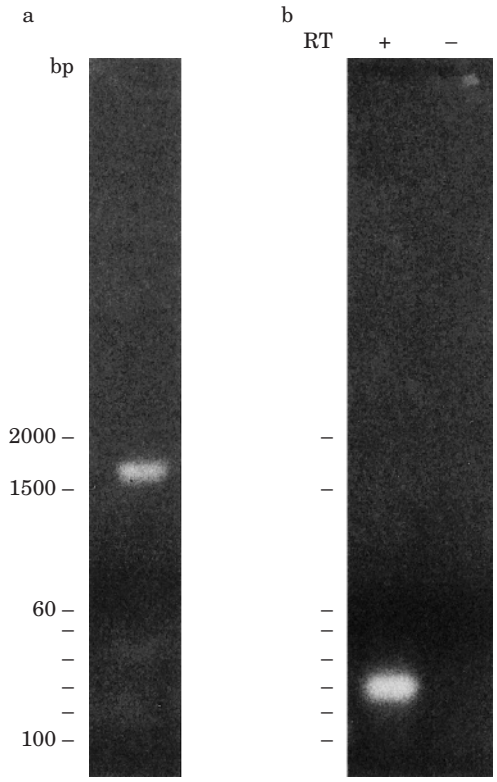

Figure 6. Characterization of the single-repeat *p270* gene of *T. vaginalis* isolate T016 and demonstration of expression. (a) PCR amplification was performed using primers localized at the distal 5′- and 3′-end of the *p270* coding region from isolate T016 genomic DNA. After PCR amplification, a 5 μl aliquot product of the PCR reaction was electrophoresed in 1% agarose. As expected, a DNA band slightly higher than 1500 bp was readily visualized by staining of agarose gels with ethidium bromide. (b) RT-PCR was performed on total RNA using two gene-specific primers as indicated in Materials and methods. The PCR products were electrophoresed in 1.5% agarose, and as expected based on the positioning of the primers, a product of ~300 bp was derived that was readily visualized by ethidium bromide staining.

the *p270* gene for isolate T016 possesses a single copy of the repeat flanked by the corresponding 5′- and 3′-end non-repeat, coding regions.

The PCR product representing the T016 *p270* gene was then ligated into pCR®2.1 and transformed into *Escherichia coli*, from which the nucleotide and amino acid sequences were derived. The *p270* gene of isolate T016 consisted of 1581 bp with the ORF translating a protein of 58.6 kDa. Alignment with the *p270* gene sequence of isolate T068-II revealed only minor differences throughout with an overall identity at the nucleotide level of 99.1%. The start codon for the *p270* T016 gene was preceded by the 12 nucleotide consensus Inr promoter-like sequence (TCATTTTTAATA) and possessed an AT-rich destabilizing element at the 3′-untranslated region found in other trichomonad protein-coding genes [20]. The 106 amino acid (318 bp) central partial repeat element contained the conserved DREGRD epitope reactive with the mAb C20A3 [15, 18], and as shown in Fig. 7, corresponded to the last, incomplete direct repeat of the TRE domain of T068-II. The 23 amino acid and 398 amino acid amino- and carboxy-terminus, respectively, flanked the partial repeat. A single nucleotide modification led to an amino acid change within the *N*-terminal part. Five amino acid changes and two new amino acids at positions 349 and 350, from the insertion of six nucleotides, were found within the C-terminal portion. The carboxy- terminus contained the putative transmembrane domain found for the other P270 proteins. These data again show the highly-conserved nature of the coding sequences of *p270* genes among isolates regardless of numbers of repeats (Fig. 2).

Expression of *p270* in *T. vaginalis* T016

Using the ^{32}P-labeled repeat cDNA as a probe, the transcript for *p270* in isolate T016 was not readily visualized by Northern analysis (data not shown), consistent with earlier reports by us [18, 20]. RT-PCR was, therefore, carried out, and as can be seen in Fig. 6(b), a PCR product of the expected size was generated. Interestingly, only use of the gene-specific antisense primer in the reverse transcription reaction led to the generation of the expected *p270* transcript fragment by PCR. This result is consistent with earlier observations by us on the low amounts of *p270* transcript in virus-free isolates [19].

Discussion

Two different types of isolates of *T. vaginalis* occur naturally and are defined on the basis of phenotypic variation for P270 [14–16, 19], the highly immunogenic protein synthesized by *T. vaginalis* [15, 23]. Phenotypic variation between surface placement and cytoplasmic expression of P270 is a property associated with infection of trichomonads by a dsRNA virus [21]. Isolates

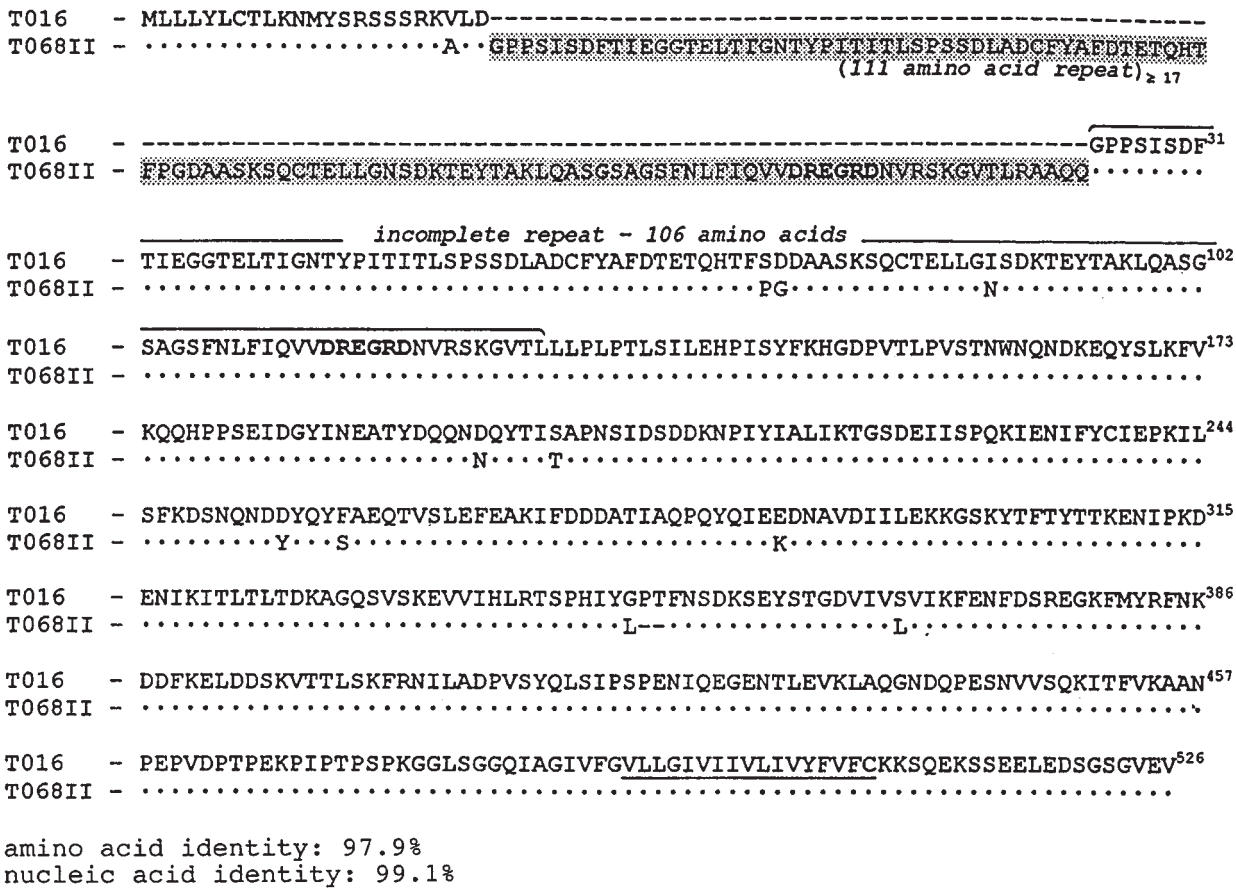

Figure 7. Derivation of the amino acid sequence for P270 of isolate T016 and alignment with the P270 of isolate T068-II. Both amino acid sequences were derived from the complete nucleotide sequences of respective *p270* genes. The shaded area consists of 111 amino acids (333 bp) of the TRE that is absent from *p270* of T016. The original 333 bp element was defined on the basis of the *Hind*III restriction site (arrow) [18, 20]. The incomplete 106 amino acid (318 bp) repeat for *p270* of T016 is bracketed. All repeats contain the DREGRD epitope (bold) recognized by mAb C20A3 [Fig. 1(a)] [15]. Unique amino acids in P270 of T068-II are shown by the substituted amino acids, and the underlined C-terminal sequence is the putative transmembrane domain identified previously [20]. Note also the two additional amino acids (PT at positions 349 and 350) from the six nucleotide insertion found within the *p270* gene of T016. For the overlapping regions, the percent identity at the amino acid and nucleotide levels were 97.9 and 99.1%, respectively.

containing organisms without dsRNA virus [15, 21] or virus-harboring parasites that lose the virus upon batch culture (virus-minus progeny) [19] synthesize up to two orders of magnitude less P270 and are unable to place P270 on the surface [14, 21]. Early examination of several other clinical isolates revealed a size polymorphism of P270 [15, 17, 19]. The experiments on the protein and gene indicated a commonality among the protein of different isolates and a gene structure of TREs comprising the high M_r protein [17, 18]. Interestingly, among patients infected with virus-harboring isolates, indirect immunofluorescence of freshly-derived organisms, monitored by detection with the mAb C20A3 [16], revealed only a minority ($\leq 5\%$) of fluorescent trichomonads with surface P270. It was further shown that mAb directed to an epitope within the TRE was cytolytic in a complement-independent fashion to trichomonads with surface P270 [13]. The differentiation of naturally-occurring isolates on the basis of phenotypic variation of P270, the size of polymorphisms of P270, and the relationship between dsRNA virus in the up-regulation of transcription and surface placement warranted a closer examination of the *p270* gene of representative clinical isolates of *T. vaginalis*.

In this report, we show the highly-conserved nature of the coding regions of *p270* genes of

fresh clinical isolates. Indeed, the *p270* genes for most of the islates that have been characterized, as shown by Southern analysis, possessed the central TRE domain [18, 20]. Further, size and identical restriction patterns among the PCR products representing both the 3′- and 5′-end non-repeat, coding regions reinforced the idea that these were highly conserved regions. That identical nucleotide sequence seen for two *p270* genes is further suggestive of the high conservation of this gene. Even more intriguing is that the gene remained highly conserved among trichomonads of individual isolates, such as NYH 286, passaged daily for over a 10-year period, suggesting an important role for this molecule that would require conservation of structure.

Given the identity of 5′- and 3′-end non-repeat, coding sequences for all *p270* genes examined to date, these suggest that the size polymorphism seen for *p270* is likely due to the overall number of the centrally-located TREs. Further, the highly-conserved nature of the amino- and carboxy-terminus of P270 from isolates with and without dsRNA virus infection is noteworthy given the fact that isolates comprised of virus-free trichomonads do not place P270 on trichomonal surfaces [21]. Furthermore, natural loss of the virus from infected *T. vaginalis* organisms, such as from long-term *in vitro* batch culture no longer yields surface placement and phenotypic variation for P270 in these organisms. These results suggest a role, albeit unknown at the present time, for the dsRNA virus to place P270 on the parasite surface.

Analyses of other isolates of *T. vaginalis* over the years by various assays consistently demonstrated a large M_r for P270 encoded by a single-copy gene [14–24]. During this study, however, PCR amplification of the 5′-end allowed for us to discover a new *p270* gene in a fresh clinical isolate. While consistent with the idea that the number of TREs constitutes the basis for P270 size polymorphisms, this is the first time that, in the evaluation of large numbers of isolates from different geographic regions, we have identified such a unique P270 protein that possessed only one partial repeat element. While the significance of this finding is unknown, it is noteworthy that the large 3′-end non-repeat, coding sequence had only minor changes in both nucleotide and amino acid sequences when compared with the T068-II *p270* gene. This too shows the highly-conserved nature of this gene apart from the number of TREs. It is noteworthy that this novel *p270* gene has been discovered in a virus-minus (Type I) isolate [14, 16], and, to date, no similar *p270* gene has been observed among other virus-harboring isolates. This will now require us to examine additional isolates, both with and without virus, to determine the frequency of this novel *p270* gene among trichomonal isolates. This will be important, as mentioned below, to ascertain the relevance, if any, of single-repeat vs multiple-repeat *p270* genes and proteins in virulence and pathogenesis.

Trichomonas vaginalis organisms are capable of evading host immune surveillance through various mechanisms, such as secretion of immunoglobulin- and complement-degrading cysteine proteinases [24, 25], shedding of antigens into their microenvironment [26], and phenotypic variation of P270 and other immunogens [14, 16, 22]. Alternating between surface vs non-surface expression of immunogens may be significant given that mAb and polyclonal serum antibody in patients to P270 is cytolytic [13]. Furthermore, expression by microbial pathogens of immunogens with TREs represents yet another mechanism of immune evasion, and surface antigens with repetitive structures have been found in many organisms [27–37]. Moreover, size polymorphism by varying the number of repeats within surface immunogens has been demonstrated, among other pathogens, in clinical isolates of *Rickettsia* spp., *Trypanosoma* spp., *U. urealyticum*, group A and B streptococci [27–31]. It is important to investigate any size polymorphism resulting from variations in repeat numbers, since this could result in alterations in both virulence of the microbial pathogen and levels of protection, depending on the protein modified [38]. This precedence has been established, as it has been shown that, for the alpha C protein of group B streptococci (GBS), variation in repeat number alters the antigenicity and protective nature of this molecule [38]. Indeed, isolation of an escape mutant with only one repeat within the alpha C protein resulted in up to two orders of magnitude greater levels of pathogenicity than GBS expressing the wild-type alpha C protein with multiple (nine) repeats [38]. Therefore, while we do not yet know the net effect on trichomonal virulence and pathogenicity resulting from the loss of the tandemly repeating central region of *p270*, as seen for isolate T016, it will be important to perform comparative evaluation of this isolate with others containing a full complement of

repeats in terms of host antibody responses, symptomatology, and *in vitro* pathogenic properties.

Materials and methods

Microorganisms

Virus-infected *T. vaginalis* isolates NYH 286, AL8, AL10, 347, and 8111, used previously and from different geographic regions [14–18], were examined along with the clinical isolates T002, T032, and T068-II [20, 24, 25]. Isolates T016 and T080 were representative of fresh clinical isolates without dsRNA virus [18, 19]. All isolates beginning with T0 designations came from the San Antonio area. Trichomonads were grown in Trypticase-yeast extract-maltose (TYM) medium supplemented with 10% heat-inactivated horse serum (HIHS) to the late-logarithmic phase of growth before harvesting [20]. Recombinant *Escherichia coli* were cultured in Luria-Betani (LB) broth or on LB agar plates with 60 μg of ampicillin per ml [39].

Immunoprecipitation (IP)

P270 proteins were immunoprecipitated as previously described [14, 15]. Briefly, 2.5×10^7 organisms were washed three times with ice-cold PBS containing 1 mM N-α-*p*-tosyl-L-lysine chloromethyl ketone, (TLCK; Sigma Chemical Co., St. Louis, MO, U.S.A.), an inhibitor of the trichomonad cysteine proteinases [24, 25] released upon solubilization. Pellets were suspended in 200 μl of NET buffer (150 mM NaCl, 5 mM EDTA, and 50 mM Tris–HCl, pH 7.2) and solubilized by addition of 25 μl 10% Zwitterionic 3-12 detergent, as before [14, 15, 23]. Then, 50 μl of mAb C20A3 was added to the detergent extract, which, following incubation overnight at 4°C with gentle shaking, the immune complexes were precipitated by addition of 50 μl 10% fixed protein A-bearing *Staphylococcus aureus*. After washing the bacteria several times in NET buffer containing 0.1% detergent, proteins were released by boiling in electrophoresis-dissolving buffer [40], and SDS-PAGE was performed using 6% acrylamide separating gels. After electrophoresis, proteins were transferred onto Trans-Blot nitrocellulose membrane (Bio-Rad Laboratories) at 170 mA for 1.5 h, and blots were then probed with the mAb C20A3 by standard procedures [14, 15, 17] for visualization of P270s.

Trichomonas vaginalis nucleic acids isolation

Total genomic trichomonad DNA was isolated by previously-described procedures [20]. The total RNA of Isolate T016 was isolated using the TRI REAGENT™ (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.) through protocols recommended by the manufacturer.

Southern analysis

Equal amounts (5.0 μg) of purified genomic DNA were digested to completion with restriction endonucleases and separated in agarose gels prior to transfer onto Zeta-probe membranes (Bio-Rad Laboratories, Richmond, CA, U.S.A.) [20]. Hybridizations were carried out with nick-translated (Promega, Madison, WI, U.S.A.) cDNA representing the 333 bp repeat as the probe [18, 20]. Blots were prehybridized (50% formamide, 120 mM Na_2HPO_4, 250 mM NaCl, 7% SDS, and 1 mM EDTA) for 4 h at 42°C. Hybridization was performed at 42°C for 18 h. Membranes were sequentially washed with 2X SSC (1X SSC is 250 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 RT for 15, 30 and 30 min, respectively.

PCR amplification and restriction analysis of amplified regions

Equal amounts (300 ng) of genomic DNA purified from the trichomonal isolates were used for PCR amplification of the targeted *p270* gene regions. For all amplifications, the hot start (94°C for 1 min) PCR procedure was performed using TaqStart Antibody (CLONTECH, Palo Alto, CA, U.S.A.) followed by three step cycles involving specific primers. Amplification of the 3′-end non-repeat, coding region employed the sense primer P1 (5′-AGGAGTCACATTACTCTTGCCATTACC) and the antisense primer M1 (5′-ACAATT-CCAAGAAGGACGCC). The target sequences were amplified in 30 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 3 min. Products were evaluated after electrophoresis of 5.0 μl aliquots of each reaction in 1% agarose gel. The 5′-end

non-repeat, coding region and the adjacent intergenic sequence was derived by PCR amplification using antisense primer M2 (5′-CGTTATCCCTACCTTCTCTATCAAC), which corresponded to the DREGRD epitope coding sequence reactive with the mAb C20A3. The sense primer P2 (5′-GAATTACTCGGAAGCAATTTAACCC) was located in the non-coding intergenic 5′-end sequence upstream of the *p270* gene start codon [20]. After the hot start, the target sequences were amplified in 30 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 3 min. Products were analysed after electrophoresis of 5.0 µl aliquots of each reaction in 1% agarose gel.

For restriction analysis, the 3′-end non-repeat, coding PCR product was purified using QIAquick PCR Purification Kit (QIAGEN Inc., Chatsworth, CA, U.S.A.). Complete digestion by restriction endonucleases was done and electrophoresis performed using 1.2% to 1.5% agarose gels to evaluate the digested DNA. In all reactions, restriction of products derived from the *p270* gene of isolate T068-II was used as a control, as done recently [20]. The products of PCR amplification of the 5′-end non-repeat region were ligated and transformed into *E. coli* INVαF′ cells (Original TA Cloning® Kit, Invitrogen, Carsbad, CA, U.S.A.). The recombinant plasmids containing inserts of the smallest PCR products were also subjected to restriction analysis.

DNA sequencing and analysis

PCR products generated by amplification of the *p270* genes of isolate T016 and of other isolates were ligated and transformed into *E. coli* INVαF′ (Original TA Cloning®). DNA sequencing was performed by the dideoxy chain termination method using the Sequenase 2.0 DNA Sequencing kit (United States Biochemical, Cleveland, OH, U.S.A.). Computer analysis of both DNA and amino acid sequences was carried out using PC/GENE (release 6.8) (IntelliGenetics, Inc., Mountain View, CA, U.S.A.) and OMEGA 1.1 (Oxford Molecular Ltd., Campbell, CA, U.S.A.).

Subcloning of the T016 *p270* gene for expression of recombinant protein

For amplification of the *p270* gene of isolate T016, the sense primer P3 (5′-TTGGATCC-A**ATG**TTACTATTATATTTATGC) was designed to introduce *Bam*HI restriction site (underlined) upstream the ATG codon (bold). The antisense primer M3 (5′-TTGG**TAC**CGACTTCAACTCCTGATCCTG) simultaneously introduced a *Kpn*I restriction site (underlined) and replaced the stop codon (bold) requirement for the expression of recombinant protein in the pTrcHis2C vector. Amplification was done after hot start in 30 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 3 min. The product was purified (QIAquick PCR Purification Kit) and digested with *Bam*HI and *Kpn*I. Further, the unidirectional ligation into double-digested vector pTrcHis2C followed by transformation into TOP10 cells was performed as recommended by the manufacturer (Invitrogen). Screening of the colonies was carried out by standard procedures [39] using anti-*myc* antibody.

Demonstration of expression by RT-PCR

The gene-specific antisense primer M4 (5′-GGTGCTGTAATAGTATACTGATG) was used in the RT-PCR reaction performed on total RNA derived from isolate T016. This same antisense primer was employed with the primer P4 (5′-AGGAGTCACATTACTCTTGCCATTACC) in the PCR amplification, which was carried out in 30 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 2 min. Products were visualized after electrophoresis in 1.5% agarose gel and staining of gels with ethidium bromide.

Nucleotide sequence accession numbers

The nucleotide sequences of the isolate T068-II 5′- and 3′-coding regions of the *p270* gene and the upstream non-coding sequence have been submitted to the GenBank data base and have been assigned GenBank accession numbers AF004356, AF004355, and AF004357, respectively (Fig. 7) [20]. The GenBank data base accession number assigned to the complete nucleotide sequence for the *p270* gene of T016 AF116276.

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