

Tpr Homologs in *Treponema paraluisuniculi* Cuniculi A Strain

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***Treponema paraluisuniculi*, the etiologic agent of rabbit venereal syphilis, is morphologically indistinguishable from *Treponema pallidum* subsp. *pallidum* (*T. pallidum*), the human syphilis treponeme, and induces similar immune responses and histopathologic changes in the infected host. Because of their high degree of relatedness, comparative studies are likely to identify genetic determinants that contribute to pathogenesis or virulence in human syphilis. The *tpr* (*Treponema pallidum* repeat) genes are believed to code for potential virulence factors. In this study, we identified 10 *tpr* homologs in *Treponema paraluisuniculi* Cuniculi A strain and determined their sequence architecture. Half of this group of paralogous genes were predicted to be nonfunctional due to the presence of frameshifts and premature stop codons. Furthermore, the immune response against the *T. paraluisuniculi* Tpr homologs in long-term-infected rabbits was studied by enzyme-linked immunosorbent assay and lymphocyte proliferation assay, showing that TprK is the only target of the antibody and T-cell responses during experimental infection and emphasizing the importance of this putative virulence factor in venereal treponematosis.**

The spirochetes are a small but diverse family of bacteria and show a wide range of host specificities. Examples of these are the easily cultivated free-living organisms (*Spirochaeta* spp.), commensal treponemes of the termite gut, the human and animal pathogens in the genera *Borrelia*, *Leptospira*, and *Brachyspira*, and the cultivable and noncultivable treponemes of humans and animals. Of the five noncultivable pathogenic treponemes, *Treponema paraluisuniculi* naturally infects rabbits but is thought not to be infectious for humans (14). In contrast, the three subspecies of *Treponema pallidum* naturally infect humans but can also experimentally infect rabbits and other mammals. *Treponema carateum* naturally infects humans but is unable to multiply in rabbits or other nonprimates (28).

Treponema paraluisuniculi is the etiologic agent of rabbit venereal syphilis. It was identified in 1913, only 8 years after *Treponema pallidum* subsp. *pallidum* (*T. pallidum* hereafter), the etiologic agent of human venereal syphilis, was discovered (3, 25). By dark-field microscopy, immunoassay, and electron microscopy, *T. paraluisuniculi* and the other pathogenic treponemes are morphologically indistinguishable (17) and appear to induce similar immune responses and histopathologic changes. Furthermore, *T. paraluisuniculi*-infected rabbits develop antibodies that can be detected with both treponemal and nontreponemal standard tests for syphilis (11, 12). Immunoblotting tests show only subtle differences between the specificities of *T. paraluisuniculi* and *T. pallidum* antisera for *T. pallidum* antigens (2, 26). Despite this apparently high level of relatedness, the pathogenic treponemes have different host specificities and cause clinically distinct diseases. Infections

with all these treponemal species are chronic, and while homologous immunity exists, there is no cross-protection between species of pathogenic treponemes and only partial cross-protection within species. These facts indicate that there are small but important differences between the virulence factors and antigens expressed by the different pathogenic treponemes.

Paralogous gene families arise during evolution by gene duplication and may yield new gene functions and new expression patterns (20–22). Initial analysis of the genome sequence of *T. pallidum* Nichols strain revealed 48 potential virulence genes, among them a novel family of 12 paralogous genes (*tpr* genes, for *Treponema pallidum* repeat) (13). The *tpr* genes account for ≈2% of the small *T. pallidum* genome. The family is divided into three subfamilies by their predicted amino acid homology: subfamily I (TprC, TprD, TprF, and TprI), subfamily II (TprE, TprG, and TprJ), and subfamily III (TprA, TprB, TprH, TprK, and TprL). The subfamily I and subfamily II proteins have conserved NH₂- and COOH-terminal regions, whereas the central domains vary sequence and length. Within subfamily I, TprC and TprD are identical in the Nichols strain, and TprF lacks a central variable domain and the conserved COOH-terminal region due to a frameshift. Subfamily III members are comparatively less homologous to each other and to the other Tpr proteins. It has been shown that TprK, TprI, and TprF are the targets of a strong humoral and cellular immune response during syphilis infection in the rabbit model, and immunization with recombinant peptides significantly alters lesion development (4, 23, 27). In addition, TprK contains a cleavable leader sequence, possesses multiple alleles in *T. pallidum* isolates, and undergoes antigenic variation during syphilis infection (6, 7, 16). This suggests an important role for the Tpr antigens during syphilis infection.

Among the several approaches used to identify virulence

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TABLE 1. Primers in the 5'- and 3'-flanking regions of the *tpr* genes

Subfamily and gene	Sense primer (5'-flanking region)	Antisense primer (3'-flanking region)	Amplicon size (bp) ^a
Subfamily I			
<i>tprC</i>	5'-GGGGGTGAGGTAGAAGTGAGA	5'-CCCTCATCCGAGACAAAAT	1,916
<i>tprD</i>	5'-AAGAGGTTTCAGGAAGCAACG	5'-ACTTCGTAGGAGCAGCAGGA	2,792
Subfamily II			
<i>tprG1</i>	5'-CAGGATTTTCCGGTTCATTG	5'-TCACGCGTTTAATGTTCTGC	3,257
<i>tprG2</i>	5'-AAGTTTGCTTTCAGAT	5'-GTACTACCTTCCCCCGGTCT	3,018
<i>tprG/I</i> hybrid	5'-CGCGTACCCACTTCTCTCTC	5'-GTACTACCTTCCCCCGGTCT	2,459
Subfamily III			
<i>tprA</i>	5'-CGTATGCTTTTACCCGCTGT	5'-TGCAACCATCTTCGATTACG	2,795
<i>tprB</i>	5'-CTTCGCGCCTAAGTTAATGC	5'-TACTCATGCGGATCCCCTAC	3,011
<i>tprK</i>	5'-TCCCCAGTTGCAGCACTAT	5'-TCGCGGTAGTCAACAATACCA	2,083–2,133 ^b
<i>tprL</i>	5'-TTCTGAGCAGCTAGGCATTG	5'-ACGGCGTCTTTCTGTACGTG	1,876
<i>tprH</i>	5'-ACGTGGCAGAGGATTTTTG	5'-TTCTCAGGAGTTTGGAACG	3,197

^a The size refers to amplicons obtained with *T. paraluiscuniculi* DNA as the template.

^b Size range of 10 *tprK* amplicons.

factors are intra- and interspecies comparisons to determine which antigens are conserved and which are variable among different strains or species (30). Weinstock et al. reported that, while *tpr* genes are highly conserved among human syphilis strains, most of the differences between the *T. pallidum* subsp. *pallidum* and *T. pallidum* subsp. *pertenue* subspecies involved the *tpr* genes (30). Despite the high level of relatedness and the natural ability to infect rabbits, *T. paraluiscuniculi* is considered not to be infectious for humans (14). The host specificity shown by this microorganism in association with the similarities to syphilitic infection make *T. paraluiscuniculi* a unique comparison species for understanding which genetic determinants contribute to pathogenesis or virulence in human syphilis. For this reason, we studied the *tpr* gene family in the rabbit treponeme to determine the sequence architecture of and immune responses to these antigens. In this study, we characterized the *tpr* gene family of *T. paraluiscuniculi*, and here we describe distinctive features of several of its members.

MATERIALS AND METHODS

***T. paraluiscuniculi* propagation and DNA extraction.** *T. paraluiscuniculi* Cuniculi A strain was provided by Paul Hardy and Ellen Nell (Johns Hopkins University, Baltimore, Md.), propagated intratesticularly in New Zealand White rabbits and harvested as described elsewhere (19). Before infection, each rabbit had been serologically tested to rule out a naturally occurring infection with *T. paraluiscuniculi*. Treponemes were extracted from infected rabbit testes at peak orchitis or 3 weeks after the infection. Collected organisms were separated from host cellular gross debris by low-speed centrifugation (250 × g for 10 min at room temperature); the supernatants were spun in a microcentrifuge for 30 min at 12,000 × g at 4°C. The pellet was resuspended in 200 μl of 1 × lysis buffer (10 mM Tris [pH 8.0], 0.1 M EDTA, 0.5% sodium dodecyl sulfate). DNA extraction was performed as previously described (5) with the QIAamp DNA minikit (Qiagen Inc., Chatsworth, Calif.), taking standard precautions to prevent cross-contamination between samples.

PCR amplification, cloning, sequencing, and sequence analysis. The *T. pallidum* genome sequence (13) was used to design primers in the 5'- and 3'-flanking regions of the Nichols *tpr* genes to amplify the corresponding *T. paraluiscuniculi* DNA regions. The primers used for amplification are listed in Table 1. Each PCR was performed in a 50-μl final volume containing 200 μM deoxynucleoside triphosphates, 50 mM Tris-HCl (pH 9.0 at 20°C), 1.5 mM MgCl₂, 20 mM NH₄SO₄, and 2.5 U of *Taq* polymerase (Promega, Madison, Wis.). Amplicons from two different PCRs with *T. paraluiscuniculi* DNA as the template were compared to minimize PCR errors. *T. pallidum* DNA was used as a positive

control for each reaction. When required, a third amplification reaction followed by cloning and sequencing was used to confirm the data from the first two reactions (*tprE* and *tprC*). The cycling conditions were denaturation at 95°C for 2 min, followed by 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min for 35 cycles in total; the final extension was 72°C for 3 min. The products were separated in 1% agarose gels and cloned into the pCRII-Topo cloning vector (Invitrogen, Carlsbad, Calif.), according to the manufacturer's instruction.

Plasmid DNA from colonies containing inserts was extracted with the Qiagen plasmid minikit (Qiagen), and at least two clones, each one from a different amplification reaction, were sequenced in both directions with the Applied Biosystems dye terminator sequencing kit (Perkin-Elmer, Foster City, Calif.) with the primer walking approach (unpublished data). Sequences were aligned with the Multiple Alignment Program (<http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>), and shading of identical bases was done with the Boxshade 3.21 program (http://www.ch.embnnet.org/software/BOX_form.html). Open reading frames (ORFs) and reading frames were determined with the NCBI ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and the Six Frames Translation (<http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html>) programs. Supplemental data is available at http://depts.washington.edu/treplab/iai72_11.htm.

Infection with *T. paraluiscuniculi*. Approximately 10⁷ organisms were used to infect six New Zealand White rabbits intratesticularly. Each rabbit had tested seronegative for prior *T. paraluiscuniculi* infection by the Venereal Disease Research Laboratory and fluorescent treponemal antibody absorption tests. To test antibody responses against recombinant Tprs, sera were collected at day 0, day 30 postinfection, and approximately every 30 days thereafter until day 270. At 270 days after infection, the rabbits were sacrificed and splenocytes were harvested to perform the lymphocyte proliferation assay.

Recombinant and synthetic peptides. The recombinant Tprs used to perform the enzyme-linked immunosorbent assays (ELISAs) and lymphocyte proliferation assays are listed in Table 2. The partial open reading frames coding for these peptides have been cloned from *T. pallidum* Nichols strain genomic DNA. Expression and purification by affinity chromatography on nickel agarose of the 6-His-tagged proteins were performed as previously described (4). TprL could not be expressed. With the exception of TprF and the peptides labeled as being from conserved regions, each recombinant peptide represents the unique central region, which contains sequences that differentiate the individual Tpr proteins; only small portions of the sequence that is conserved among members of the same subfamily are present in the peptides.

The subfamily III recombinant proteins (TprA N-terminal and C-terminal, TprB-central, TprH-central, and TprK-central) have virtually no overlap, while Tpr I-central, TprE-central, TprG-central, and TprJ-central each contain 23 amino acids of the NH₂-terminal conserved region; TprI-central also contains 21 amino acids of the COOH-terminal conserved region. TprF, in contrast, contains 262 amino acids of the NH₂-terminal constant region of subfamily I in addition to 87 amino acids representing the central region. TprD2-central is an allele-specific peptide. The peptides representing the conserved amino terminus and

TABLE 2. Recombinant peptides used for antibody response and lymphocyte proliferation assays

Subfamily and peptide	Amino acid sequence limits	Size (no. of residues)	Identity ^a with <i>T. paraluisuniculi</i> predicted ORFs
Subfamily I			
5'-subfamily 1	YAGVLT. . . . QHQDPA	267	98.6% identity with TprC and TprD before frameshift
TprF (N-terminal and central region)	YAGVLT. . . . GTGGAC	349	98.6% identity with TprC and TprD before frameshift
TprD2-central	TSLGD. . . . WLQFTY	90	96% with TprC and TprD
3'-subfamily I (A) ^b	GFLRLE. . . . TKSGDP	86	81% with TprC and TprD
3'-subfamily I (B) ^b	RIPGLS. . . . IAESIW	76	82% with TprC and TprD
TprI-central	RLTLEP. . . . YTHLLT	211	80% with TprG/I hybrid
Subfamily II			
TprG-central	RLTLEP. . . . DLIPKT	219	85.3% with TprG1 and TprG2
3'-Subfamily II	LFTAQW. . . . GVTLSV	202	89.3% with TprG1; 89.6% with TprG2
TprE-central	RLTLEP. . . . QQTVA A	191	9.5% with TprG1 and TprG2
TprJ-central	RLTLEP. . . . MRTEIT	226	56.6% with TprG1 and TprG2
Subfamily III			
TprA (N-terminus)	MRLVLG. . . . NCGTRR	253	91.6%
TprA (C-terminus)	MGLVVT. . . . GCKITW	368	90%
TprB-central	RLTLSP. . . . SLSKLV	194	99.4%
TprH-central	RITLTP. . . . YTHLID	180	98.8%
TprK-central ^c	IEGYAE. . . . DTSFLE	315	84%
TprL ^d	NA	NA	NA

^a To define identity, *T. pallidum* recombinant peptide sequences were aligned with their *T. paraluisuniculi* homologues and the value was calculated as (number of identical amino acids in identical position/recombinant peptide size) × 100.

^b 3'-subfamily I A and B are partially overlapping peptides encompassing positions 313 to 395 (peptide A) and 379 to 454 (peptide B) of *T. paraluisuniculi* TprC and TprD (B ORF, Fig. 1), respectively.

^c The TprK 1.1 clone was used for this calculation.

^d Recombinant peptide not available (NA).

carboxyl terminus of subfamilies I and II are labeled 5'-conserved and 3'-conserved, respectively. We were unable to produce the 5'-conserved peptide for subfamily II, and the 3'-conserved peptide of subfamily I had to be produced in two smaller fragments, as expression of full-length peptide appeared to be toxic to *Escherichia coli*. The percent identity between the Nichols-based recombinant antigens and *T. paraluisuniculi* Tprs is indicated in Table 2.

Overlapping 20-amino-acid-long synthetic peptides were synthesized based on the Seattle Nichols strain TprK sequence (GenBank accession number AF194369) as described elsewhere (24). Thirty-nine peptides with a 10-amino-acid overlap, spanning the entire mature TprK sequence (amino acids 30 through 349) (16) (see Fig. 6 and data not shown) were used in lymphocyte proliferation assays. Additionally, the conserved carboxyl terminus (amino acids 425 to 478, listed as peptide 40) was cloned and expressed as a recombinant protein as described previously (24).

ELISAs. The appropriate purified recombinant Tpr antigen (0.5 µg per well) in phosphate-buffered saline (PBS) containing 0.1% sodium azide and 0.1% sodium dodecyl sulfate was used to coat the wells of a 96-well ELISA plate (EIA II Plus Microplate; ICN, Irvine, Calif.). The plates were incubated at 37°C for 2 h and subsequently at 4°C overnight. The wells were then washed three times with PBS, blocked by incubation for 1 h at room temperature with 200 µl of 3% nonfat milk-PBS/well, and washed again.

Rabbit sera collected from six animals prior to infection and at days 30, 60, 90, 130, 160, 190, 230, and 270 postinfection were pooled for each time point and adsorbed with a lysate from the *E. coli* strain used to express the recombinant antigens. This lysate of the *E. coli* strain expressing an unrelated *Trypanosoma cruzi* recombinant protein (SA85 1.1) was used to remove cross-reacting antibody against possible *E. coli* contaminants and the histidine tag added to the recombinant peptides during expression (27). Ten microliters of each adsorbed pool was diluted 1:20 in 1% nonfat milk-PBS plus 0.05% Tween 20 (Sigma) prior to being dispensed into wells. Only one concentration of serum was tested. One hundred microliters of diluted primary antibody was incubated for 1 h at room temperature, and then the wells were washed three times with PBS. One hundred microliters of secondary antibody solution, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma, St. Louis, Mo.), diluted 1:2,000 in 1% nonfat milk-PBS-0.05% Tween 20, was then added to each well, and the plates were incubated for 1 h at room temperature before repeating the washing step. Finally, the plates were developed for 45 min after addition of 50 µl of 1-mg/ml

para-nitrophenyl phosphate (Sigma) to each well and read at 405 nm on a Multiskan MC plate reader (Titertek, Huntsville, Ala.). The mean of background readings (from preimmune rabbit sera) was subtracted from the mean of triplicate experimental wells for each antigen. The reported data are the mean ± standard error of the mean for each antigen per time point.

Lymphocyte proliferation assays. Three uninfected and five Cuniculi A strain-infected rabbits (day 270 postinfection) were sacrificed, and splenic lymphocytes were harvested and cultured as previously described (1, 19) in RPMI medium supplemented with glutamine (2 mM final concentration), penicillin (100 U/ml final concentration), streptomycin (100 µg/ml final concentration), and heat-inactivated normal rabbit serum (1% final concentration). Approximately 5 × 10⁵ cells in 200 µl of culture medium were plated in quadruplicate for each animal and each antigen in flat-bottomed 96-well plates (Costar, Cambridge, Mass.) and incubated at 37°C in a 5% CO₂ atmosphere. Recombinant proteins were added at 2 and 10 µg/ml final concentrations, and synthetic peptides were added at a 25 µg/ml final concentration; 10 µl/well of a sonicated suspension of *T. pallidum* was used as the peptide-specific positive control, and 0.5 µg/well (as determined by titration) of concanavalin A (ICN) used as the positive T-cell control. PBS was used to measure background reactivity. Three days after exposure to recombinant peptide or controls, cells were pulsed with 0.5 µCi of [³H]thymidine per well and harvested after 24 h to measure tritiated thymidine incorporation. The geometric mean of quadruplicate wells with no antigens (determined as background value) was subtracted from the geometric means of the quadruplicate experimental wells. The reported data represent the geometric means (± standard error) of values for each antigen for five rabbits.

Nucleotide sequence accession numbers. The sequences identified in this study can be found at GenBank under accession numbers AY685232 to AY685240 (*tprA*, *tprB*, *tprH*, *tprL*, *tprC*, *tprD*, *tprG/I*, *tprG1*, and *tprG2*, respectively) and AY685241 to AY685250 for *tprK* (clones 1.1 to 1.5 and 2.1 to 2.5, respectively).

RESULTS

Identification of *tpr* homologs in *T. paraluisuniculi*. All *tpr* loci defined in the Nichols genome sequence were analyzed in the corresponding chromosomal locations in *T. paraluisuniculi*.

TABLE 3. Characteristics of *T. paraluiscuniculi* *tpr* genes and predicted proteins

<i>T. paraluiscuniculi</i> Tpr	Closest Nichols strain homologue	Primer set ^a	ORF length ^b (bp)	Predicted size (kDa)	Signal peptide ^c	Predicted cellular location ^d
TprG/I hybrid		<i>tprG</i> (sense), <i>tprF</i> (antisense)				
Reading frame A	TprG (amino-terminal region)		777	27.7	U	IM
Reading frame B	TprI (central and carboxyl-terminal regions)		1,344			
Subfamily I						
TprC		<i>tprC</i>				
Reading frame A	TprD2 ^e		591	21.4	C	P/OM
Reading frame B	TprD2		1,413			
TprD		<i>tprD</i>				
Reading frame A	TprD2		591	21.4	C	P/OM
Reading frame B	TprD2		1,413			
Subfamily II						
TprG1		<i>tprE</i>				
Reading frame A	TprG		753	27.7	U	IM
Reading frame B		1,752				
TprG2		<i>tprJ</i>				
Reading frame A	TprG		753	27.7	U	IM
Reading frame B	TprG		1,752			
Subfamily III						
TprA	TprA	<i>tprA</i>	1,797	65.9	U	IM
TprB	TprB	<i>tprB</i>	1,935	69.5	N	Cy
TprH	TprH	<i>tprH</i>	2,082	76.0	N	Cy
TprK ^f	TprK	<i>tprK</i>	1,518	57.7	C	P/OM ^g
TprL	TprL	<i>tprL</i>	1,341	48.8	N	Cy

^a Indicates which *tpr* gene was targeted for amplification. In the Nichols strain of *T. pallidum*, each set recognizes the flanking regions of the gene indicated. Reading frames A and B indicate the two ORFs generated by the frameshift. No predictions (size, signal peptide, and cellular localization) are reported for the B ORFs due to the lack of evidence that these regions are actually transcribed or translated.

^b Total length includes stop codon.

^c Signal peptide predicted by Psort (<http://psort.nibb.ac.jp/>). U, uncleavable; C, cleavable; N, none.

^d Location of the predicted protein in the bacterial cell, predicted by Psort. OM, outer membrane; IM, inner membrane; P, periplasmic; Cy, cytoplasmic.

^e The *tprD2* allele is not present in the Nichols strain genome. However, it was reported in several strains of *T. pallidum* subsp. *pallidum* (8).

^f The TprK 1.1 clone was used for this prediction.

^g Prediction made with the translational start site for Nichols TprK (4, 15) at position 26 of the sequence indicated as TprK-N in Fig. 7.

niculi Cuniculi A strain. The DNA and predicted amino acid sequences were aligned with the *T. pallidum* Nichols strain homologs, followed by BLAST searches with the *T. paraluiscuniculi* sequences as the query to find additional regions of homology with other *tpr* sequences available in the databases. Overall, we identified three major subfamilies and one hybrid ORF. These are described in Table 3.

Subfamily I in *T. pallidum* is composed of four members, *tprC*, *tprD*, *tprF*, and *tprI*. In the Cuniculi A strain, however, we identified only the *tprC* and *tprD* alleles. These loci contain identical sequences (Fig. 1), which are highly homologous to the *T. pallidum* *tprD2* allele described previously (8). However, these ORFs do not encode full-length TprD2 amino acid sequences. Instead, there is an identical frameshift in each ORF due to the presence of an extra guanosine at position 439 of the coding sequence (amino acid position 147 of the Cuniculi A TprC and TprD ORFs; Fig. 1). This introduces a premature termination, which generates a putative protein sequence of only 196 amino acids instead of 597 (expected in the absence of a frameshift). However, both amino acid sequences (upstream and downstream of the frameshift) are included in the alignments for comparison purposes. The region corresponding to the Nichols *tprI* locus is absent in *T. paraluiscuniculi*, but its

3'-flanking region is nonetheless preserved. A hybrid ORF containing the central domain and carboxyl terminus of a *tprI*-like sequence was found in the region of the Nichols *tprF* locus (described below as a *tprG/I* hybrid).

The DNA regions in Cuniculi A corresponding to the Nichols *tprE* and *tprJ* loci were found to contain two identical sequences with a complex pattern of homology to the Nichols subfamily II *tprs* (Fig. 2 and Fig. 4A). Their 5' end aligns with the conserved 5' end of the subfamily II *tprs* (*tprE*, *tprG*, *tprJ*); the central regions align with the central portion of the Nichols *tprG* and their 3' end with the corresponding fragment of the Nichols *tprJ* (Fig. 2 and 4B). Despite the complex nature of the sequence composition, they show the highest homology to Nichols *tprG*. For this reason, they are called here *tprG1* and *tprG2*. Similar to the *tprC* and *tprD* alleles, these two ORFs have an extra guanosine in position 653 of the coding sequence, which also creates a frameshift (amino acid position 217 of Cuniculi A TprG1 and TprG2 ORFs; Fig. 2) and a premature stop in the predicted amino acid sequences. There is no *tprE* homolog.

In *T. paraluiscuniculi*, a single *tprG/I* hybrid ORF replaces the loci occupied by *tprG* and *tprF* in the Nichols strain (Fig. 3). It is unique in that the 5' end is homologous to the Nichols

TprC-C	A	1	MGRQVMQAGVVRAGVVCAASGYAVLTPQVSGTAQLQWGIAPQKNPHTDPGKHTHGFRRTTNSLTI SLPLVSKH <th>TRRGEA</th>	TRRGEA
TprD-C	A	1	MGRQVMQAGVVRAGVVCAASGYAVLTPQVSGTAQLQWGIAPQKNPHTDPGKHTHGFRRTTNSLTI SLPLVSKH <th>TRRGEA</th>	TRRGEA
TprD2		1	MGRQVMQAGVVRAGVVCAASGYAVLTPQVSGTAQLQWGIAPQKNPHTDPGKHTHGFRRTTNSLTI SLPLVSKH <th>TRRGEA</th>	TRRGEA
			frameshift	
			*	
TprC-C	A	81	RS GVWAQLQLKDLAVELASSKSSTALSFTKPTASFOATLHCYGA Y LT VG -TSPSCVVNFAQL W EPFVDPCLFRKEHSLCP	
TprD-C	A	81	RS GVWAQLQLKDLAVELASSKSSTALSFTKPTASFOATLHCYGA Y LT VG -TSPSCVVNFAQL W EPFVDPCLFRKEHSLCP	
TprC-C	B	1	QWVPVLPV W LTSPSCGNPLS TRAYSEKNTRYAP	
TprD-C	B	1	QWVPVLPV W LTSPSCGNPLS TRAYSEKNTRYAP	
TprD2		81	RS GVWAQLQLKDLAVELASSKSSTALSFTKPTASFOATLHCYGA Y LT VG -TSPSCVVNFAQL W EPFV TRAYSEK DT RYAP	
TprC-C	A	160	WFLRLRGKTRLP GP QCGKQRSRCGHRF PLL PFQWCLG	
TprD-C	A	160	WFLRLRGKTRLP GP QCGKQRSRCGHRF PLL PFQWCLG	
TprC-C	B	34	GF --- SGS ---GAKLGYQAHNVGNSGVDVDIGFLSFLSNGAWDSTD T THSKYGF G ADATLSYGVDRQRL L TLELAG N AT	
TprD-C	B	34	GF --- SGS ---GAKLGYQAHNVGNSGVDVDIGFLSFLSNGAWDSTD T THSKYGF G ADATLSYGVDRQRL L TLELAG N AT	
TprD2		160	GF --- SGS ---GAKLGYQAHNVGNSGVDVDIGFLSFLSNGAWDSTD T THSKYGF G ADATLSYGVDRQRL L TLELAG N AT	
TprC-C	B	107	LE QNYLKGTEDPK N ENKTALLWGVGGRLTLE P GAGFRFSFALDAGNQHQSDAQFYARMAPSQ R VEV I NSLGD T LLT S PK	
TprD-C	B	107	LE QNYLKGTEDPK N ENKTALLWGVGGRLTLE P GAGFRFSFALDAGNQHQSDAQFYARMAPSQ R VEV I NSLGD T LLT S PK	
TprD2		233	LE QNYLKGTEDPK N ENKTALLWGVGGRLTLE P GAGFRFSFALDAGNQHQSDAQFYARMAPSQ R VEV I NSLGD T LLT S PK	
TprC-C	B	187	QD VVSFFVQELSKGS L LEKAGLV T LLAQRTIVSLASTGGYLRHLNGK G LEINMRLIEQ Q KNPDAR M RTALFIS W LQF T YT	
TprD-C	B	187	QD VVSFFVQELSKGS L LEKAGLV T LLAQRTIVSLASTGGYLRHLNGK G LEINMRLIEQ Q KNPDAR M RTALFIS W LQF T YT	
TprD2		313	QD VVSFFVQELSKGS L LEKAGLV T LLAQRTIV G LASGGYLRHLNGK G LEINMRLIEQ Q KNPDAR M RTALFIS W LQF T YT	
TprC-C	B	267	K TLNIDALLGMQWR W LS S GIYFATAGT N IFGERVFFKNQADHFD F AG F LK L E T KS G D P Y T H L L T GL N AG V E A R V Y I PL T Y	
TprD-C	B	267	K TLNIDALLGMQWR W LS S GIYFATAGT N IFGERVFFKNQADHFD F AG F LK L E T KS G D P Y T H L L T GL N AG V E A R V Y I PL T Y	
TprD2		393	K TLNIDALLGMQWR W LS S GIYFATAGT N IFGERVFFKNQADHFD F AG F LK L E T KS G D P Y T H L L T GL N AG V E A R V Y I PL T Y	
TprC-C	B	347	I F Y KNNGGAQ Y KGSNSD G VINT P ILSKAWCS Y RI P L G SHAW L AP H TS V L W AT N RF N H N Q S GD A LL R E H AL Q Y Q V G L T F S P	
TprD-C	B	347	I F Y KNNGGAQ Y KGSNSD G VINT P ILSKAWCS Y RI P L G SHAW L AP H TS V L W AT N RF N H N Q S GD A LL R E H AL Q Y Q V G L T F S P	
TprD2		473	I F Y KNNGGAQ Y KGSNSD G VINT P ILSKAWCS Y RI P L G SHAW L AP H TS V L W AT N RF N H N Q S GD A LL R E H AL Q Y Q V G L T F S P	
TprC-C	B	427	F E K VELSAQ W E Q GV L AD A P Y M G IT E SI W S E R H F G T F V C G M K V T W	
TprD-C	B	427	F E K VELSAQ W E Q GV L AD A P Y M G IT E SI W S E R H F G T F V C G M K V T W	
TprD2		553	F E K VELSAQ W E Q GV L AD A P Y M G IT E SI W S E R H F G T F V C G M K V T W	

FIG. 1. Comparison of the TprC and TprD loci protein sequences of *T. paraluisuniculi* with the TprD2 sequence of *T. pallidum*. TprC-C, TprD-C indicate the TprC and TprD alleles of *T. paraluisuniculi*, respectively. A, NH₂-terminal region; B, central unique and COOH-terminal regions. Identical residues are indicated by black shading, and nonsynonymous changes are indicated in gray or no shading, respectively. *, frameshift position in *T. paraluisuniculi* Tprs (TprC-C and TprD-C A ORFs, amino acid position 147); TprD2, sequence from GenBank (Accession number AF217541).

subfamily II 5' conserved region, and the central unique region and the 3' end contain a *tprI*-like sequence (Fig. 4B). The presence of a complete central region and a 3' end with a stop codon as predicted in the Nichols *tprI* ORF rules out the possibility that the sequence in the *G/I* hybrid is *tprF* (*tprF* contains a large deletion in the central region, a frameshift and a premature stop). Interestingly, as seen in the Cuniculi A *tprD2* alleles and in the *tprG1* and *tprG2* ORFs, this hybrid molecule also has a frameshift at nucleotide position 653 (amino acid position 217 of TprG/I, A ORF; Fig. 3), which introduces a premature stop in the predicted protein sequence (Fig. 3).

As in the human treponeme, Cuniculi A subfamily III is composed of five members (*tprA*, *tprB*, *tprH*, *tprK*, and *tprL*)

with poor homology to each other and to the other predicted Cuniculi A *tprs*. *tprH* and *tprB* are virtually identical to their *T. pallidum* homologs except for scattered nonsynonymous single-nucleotide polymorphisms which introduce four amino acid changes in TprB and five in TprH (unpublished data). *tprA* in the Nichols strain contains an authentic frameshift at nucleotide number 712 (amino acid position 237 of Nichols TprA ORF; Fig. 5) located in a region containing three dinucleotide repeats (CT) at positions 706 to 711 in the ORF. In contrast, the *tprA* ORF in *T. paraluisuniculi* contains four CT dinucleotide repeats, which revert the frameshift and generate a sequence encoding a full-length ORF.

The presence of dinucleotide repeats is frequently associated with regulatory mechanisms of gene expression, where the

TprG1-C A 1 MGCMRWGSVLCVVVGVGASGGVVGQEFSPKLTGSALEWGISYKGVGSHDQAPGAVMGTGPYNLKHGFRRTNTVGVSP
 TprG2-C A 1 MGCMRWGSVLCVVVGVGASGGVVGQEFSPKLTGSALEWGISYKGVGSHDQAPGAVMGTGPYNLKHGFRRTNTVGVSP
 TprG-N 1 MGCMRWGSVLCVVVGVGASGGVVGQEFSPKLTGSALEWGISYKGVGSHGQAPGAVMGTGPYNLKHGFRRTNTVGVSP
 TprJ-N 1 MGCMRWGSVLCVVVGVGASGGVVGQEFSPKLTGSALEWGISYKGVGSHGQAPGAVMGTGPYNLKHGFRRTNTVGVSP
 TprE-N 1 MGCMRWGSVLCVVVGVGASGGVVGQEFSPKLTGSALEWGISYKGVGSHGQAPGAVMGTGPYNLKHGFRRTNTVGVSP

TprG1-C A 81 LVMRTTHTRRGQHPALYAELEKVALDQADLSQGKAGDFKRRKGVKVEATLHCYGAYLITGKNPTFLTNFARLWKPWVTAQYQ
 TprG2-C A 81 LVMRTTHTRRGQHPALYAELEKVALDQADLSQGKAGDFKRRKGVKVEATLHCYGAYLITGKNPTFLTNFARLWKPWVTAQYQ
 TprG-N 81 LVMRTTHTRRGQHPALYAELEKVALDQADLSQGKAGFAVKRRKGVKVEATLHCYGAYLITGKNPTFLTNFARLWKPWVTAQYQ
 TprJ-N 81 LVMRTTHTRRGQHPALYAELEKVALDQADLSQGKAGFAVKRRKGVKVEATLHCYGAYLITGKNPTFLTNFARLWKPWVTAQYQ
 TprE-N 81 LVMRTTHTRRGQHPALYAELEKVALDQADLSQGKAGFAVKRRKGVKVEATLHCYGAYLITGKNPTFLTNFARLWKPWVTAQYQ

frameshift
*

TprG1-C A 161 EDEVQYAPGFGGLGKVGYRAQDIGSGVSLDVGFLSFAASNGAWDSTDITHSKYGFGSRLEANVCACRTPSVHGRACQQC
 TprG2-C A 161 EDEVQYAPGFGGLGKVGYRAQDIGSGVSLDVGFLSFAASNGAWDSTDITHSKYGFGSRLEANVCACRTPSVHGRACQQC
 TprG1-C B 1 -----AARLGI EHRTLGA VGSALMMWGFSPLPLTVPGIVLTPRTASMAIGADLKLMYARAGHPLCTVELASNV
 TprG2-C B 1 -----AARLGI EHRTLGA VGSALMMWGFSPLPLTVPGIVLTPRTASMAIGADLKLMYARAGHPLCTVELASNV
 TprG-N 161 EDAVQYAPGFGGLGKVGYRAQDIGSGVSLDVGFLSFAASNGAWDSTDITHSKYGFGADLKLMYARAGHPLCTVELASNV
 TprJ-N 161 EDAVQYAPGFGGLGKVGYRAQDIGSGVSLDVGFLSFAASNGAWDSTDITHSKYGFGADLKLMYARAGHPLCTVELASNV
 TprE-N 161 EDAVQYAPGFGGLGKVGYRAQDIGSGVSLDVGFLSFAASNGAWDSTDITHSKYGFGADLKLMYARAGHPLCTVELASNV

TprG1-C A 231 YARRIPHRCAEGRKQSEQG
 TprG2-C A 231 YARRIPHRCAEGRKQSEQG
 TprG1-C B 68 TLEDGYLIGAQKANNQNKDKLLWNVGGRLTLEPGA GFRFSFALDAGNQHQSAODFQNRTORAQSELTALSNNLFQGESQ
 TprG2-C B 68 TLEDGYLIGAQKANNQNKDKLLWNVGGRLTLEPGA GFRFSFALDAGNQHQSAODFQNRTORAQSELTALSNNLFQGESQ
 TprG-N 241 TLEDGYLIGAQKANNQNKDKLLWNVGGRLTLEPGA GFRFSFALDAGNQHQSAODFQNRTORAQSELTALSNNLFQGESQ
 TprJ-N 241 TLEDGYLIGAQKANNQNKDKLLWNVGGRLTLEPGA GFRFSFALDAGNQHQSAODFQNRTORAQSELTALSNNLFQGESQ
 TprE-N 241 TLEDGYLIGAQKANNQNKDKLLWNVGGRLTLEPGA GFRFSFALDAGNQHQSEATAAMRTERTREARQEVALLAIFTHAQ

TprG1-C B 148 KQEAWLDEYAKKVLDAVTAATETAIC-SRGNAYITAVSNVKVTP----PVAATLLNLKVFIT----DPPTPSPLPALP
 TprG2-C B 148 KQEAWLDEYAKKVLDAVTAATETAIC-SRGNAYITAVSNVKVTP----PVAATLLNLKVFIT----DPPTPSPLPALP
 TprG-N 321 KQEAWLDEYAKKVLDAVTAATETAIC-SRGNAYITAVSNVKVTP----PVAATLLNLKVFIT----DPPTPSPLPALP
 TprJ-N 321 KQEAWLDEYAKKVLDAVTAATETAIC-SRGNAYITAVSNVKVTP----PVAATLLNLKVFIT----DPPTPSPLPALP
 TprE-N 321 KQEAWLDEYAKKVLDAVTAATETAIC-SRGNAYITAVSNVKVTP----PVAATLLNLKVFIT----DPPTPSPLPALP

TprG1-C B 218 AFS----LMGOVLLQYGVQVVKGF EQVQTQIVAEINOKVQAVGERSRDAACTFSNGLAQTVAARIADKLLMPHIGN-IR
 TprG2-C B 218 AFS----LMGOVLLQYGVQVVKGF EQVQTQIVAEINOKVQAVGERSRDAACTFSNGLAQTVAARIADKLLMPHIGN-IR
 TprG-N 391 AFS----LMGOVLLQYGVQVVKGF EQVQTQIVAEINOKVQAVGERSRDAACTFSNGLAQTVAARIADKLLMPHIGN-IR
 TprJ-N 392 AFS----LMGOVLLQYGVQVVKGF EQVQTQIVAEINOKVQAVGERSRDAACTFSNGLAQTVAARIADKLLMPHIGN-IR
 TprE-N 394 AFS----LMGOVLLQYGVQVVKGF EQVQTQIVAEINOKVQAVGERSRDAACTFSNGLAQTVAARIADKLLMPHIGN-IR

TprG1-C B 292 SLFNDFNQOATLRQDLTDL-----IPKLTAEATKFFTEGOTFVTEEVKKTDAALDAGQIQAQIQLRASAWRAFLMGVS
 TprG2-C B 292 SLFNDFNQOATLRQDLTDL-----IPKLTAEATKFFTEGOTFVTEEVKKTDAALDAGQIQAQIQLRASAWRAFLMGVS
 TprG-N 465 SLFNDFNQOATLRQDLTDL-----IPKLTAEATKFFTEGOTFVTEEVKKTDAALDAGQIQAQIQLRASAWRAFLMGVS
 TprJ-N 467 SLFNDFNQOATLRQDLTDL-----IPKLTAEATKFFTEGOTFVTEEVKKTDAALDAGQIQAQIQLRASAWRAFLMGVS
 TprE-N 467 HTINGKSYAAHI-GSLVSLATNRALPTIRQRVEQAVCENIRRNNAVVOQCAQTLLTSOEBLEKAWYSLFVPTTEMLVIGAG

TprG1-C B 367 AVCLYLDTYNVAFDALFTAQWKWLSGSIYFATAPANVFGTRVLDNTIASCGDFAGFLKLETSGDPYTHLLTGLDAGVET
 TprG2-C B 367 AVCLYLDTYNVAFDALFTAQWKWLSGSIYFATAPANVFGTRVLDNTIASCGDFAGFLKLETSGDPYTHLLTGLDAGVET
 TprG-N 540 AVCLYLDTYNVAFDALFTAQWKWLSGSIYFATAPANVFGTRVLDNTIASCGDFAGFLKLETSGDPYTHLLTGLDAGVET
 TprJ-N 542 AVCLYLDTYNVAFDALFTAQWKWLSGSIYFATAPANVFGTRVLDNTIASCGDFAGFLKLETSGDPYTHLLTGLDAGVET
 TprE-N 546 AVCLYLDTYNVAFDALFTAQWKWLSGSIYFATAPANVFGTRVLDNTIASCGDFAGFLKLETSGDPYTHLLTGLDAGVET

TprG1-C B 447 RVYIPLTHDLYKNNNGMPLPSGGSSGHSIPLIVSRAWCYSYRIPVQDYGVWKPSVTVHASTNRAHLNTPAAGGAVGATYLT
 TprG2-C B 447 RVYIPLTHDLYKNNNGMPLPSGGSSGHSIPLIVSRAWCYSYRIPVQDYGVWKPSVTVHASTNRAHLNTPAAGGAVGATYLT
 TprG-N 620 RVYIPLTHDLYKNNNGMPLPSGGSSGHSIPLIVSRAWCYSYRIPVQDYGVWKPSVTVHASTNRAHLNTPAAGGAVGATYLT
 TprJ-N 622 RVYIPLTHDLYKNNNGMPLPSGGSSGHSIPLIVSRAWCYSYRIPVQDYGVWKPSVTVHASTNRAHLNTPAAGGAVGATYLT
 TprE-N 626 RVYIPLTHDLYKNNNGMPLPSGGSSGHSIPLIVSRAWCYSYRIPVQDYGVWKPSVTVHASTNRAHLNTPAAGGAVGATYLT

TprG1-C B 527 KEYCAQLRAGISASLIEKTVFSLDWEQGMLSDVPYLLVSECLTQGI GRIVCGVTLSSW
 TprG2-C B 527 KEYCAQLRAGISASLIEKTVFSLDWEQGMLSDVPYLLVSECLTQGI GRIVCGVTLSSW
 TprG-N 700 KEYCAQLRAGISASLIEKTVFSLDWEQGMLSDVPYLLVSECLTQGI GRIVCGVTLSSW
 TprJ-N 702 KEYCAQLRAGISASLIEKTVFSLDWEQGMLSDVPYLLVSECLTQGI GRIVCGVTLSSW
 TprE-N 706 KEYCAQLRAGISASLIEKTVFSLDWEQGMLSDVPYLLVSECLTQGI GRIVCGVTLSSW

FIG. 2. Alignment of the subfamily II Tpr homologs of *T. paraluiscuniculi* and *T. pallidum*. TprG1-C and TprG2-C, *T. paraluiscuniculi* sequences. TprG-N, TprJ-N, and TprE-N, *T. pallidum* Nichols strain sequences. A, open reading frame A (NH₂-terminal region); B, open reading frame B (central unique and COOH-terminal regions). *, frameshift position in *T. paraluiscuniculi* Tprs (TprG1-C and TprG2-C A ORFs, amino acid position 217).

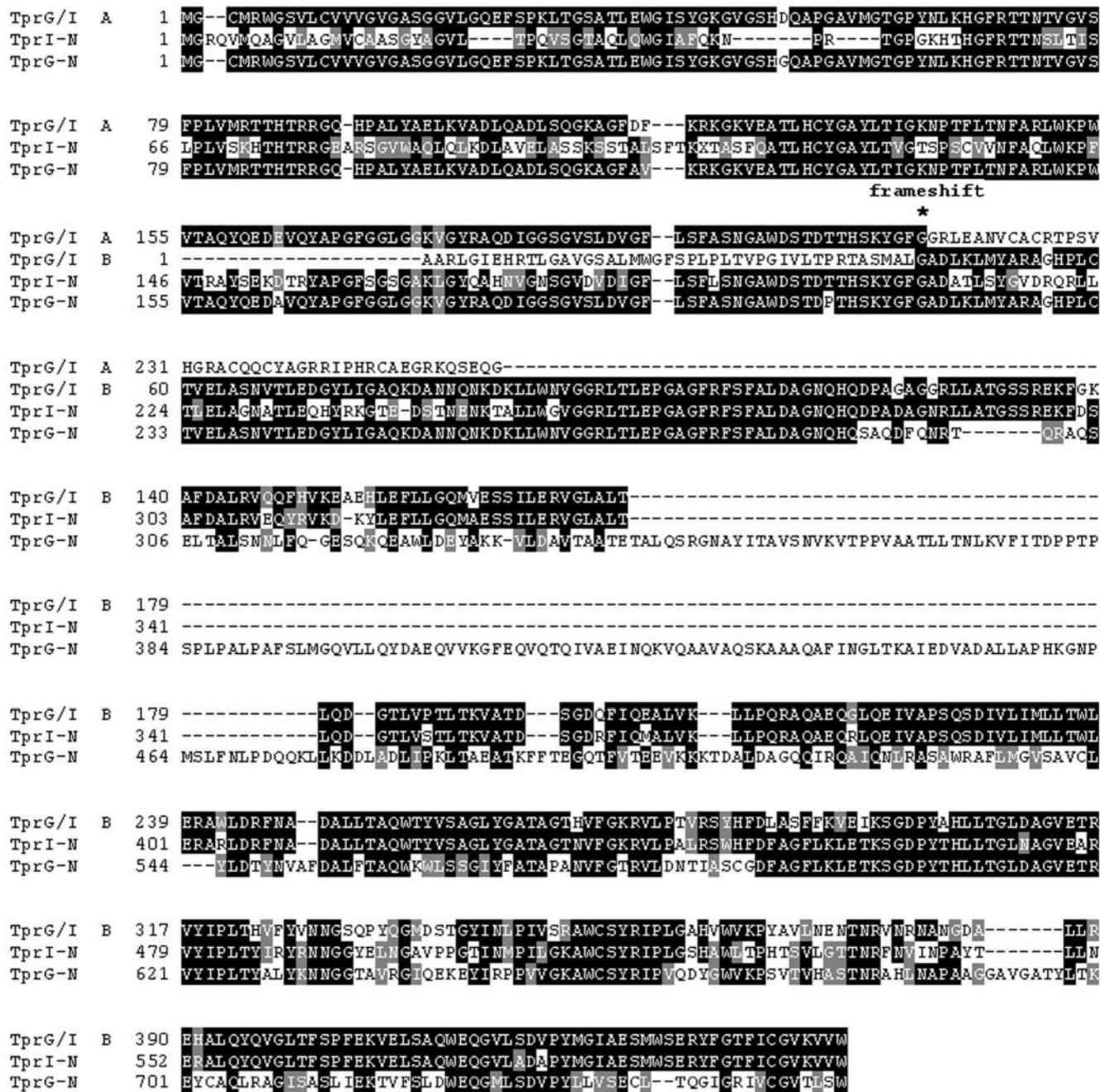


FIG. 3. Alignment of the *T. paraluisuniculi* TprG/I hybrid and the TprG and TprI homologs of *T. pallidum*. TprG/I, *T. paraluisuniculi* TprG/I hybrid. TprG-N, TprI-N, *T. pallidum* Nichols strain sequences. A, open reading frame A (NH₂-terminal region); B, open reading frame B (central unique and COOH-terminal regions). *, frameshift position in *T. paraluisuniculi* TprG/I (amino acid position 217 of TprG/I A ORF).

number of repeats varies according to an on or off state to achieve phase variation of gene expression. To see whether variation in the number of dinucleotide repeats is due to such a mechanism as an explanation for the absence of a frameshift and premature termination in Cuniculi A *tprA*, we sequenced 15 more clones of a DNA region of approximately 800 bp containing the dinucleotide repeats (data not shown). All sequences confirmed the absence of a frameshift in the rabbit treponeme *tprA* homolog. We are aware, however, that

changes in the number of repeats that occur at low frequencies may not be detected with this approach. Alignment of the Nichols and Cuniculi A *tprL* homologs showed conserved 5' and 3' ends with a different central region of 366 nucleotides (122 amino acids, positions 131 to 252 of Cuniculi A TprL; Fig. 6). This unique region in Cuniculi A has no sequence homology in the *T. pallidum* databases.

We have shown previously that the TprK antigen in *T. pallidum* is heterogeneous within and among *T. pallidum* isolates.

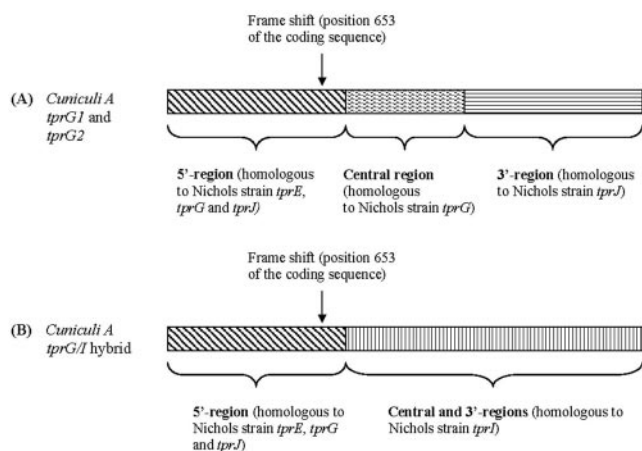


FIG. 4. Schematic representation of *T. paraluiscuniculi* subfamily II members found in loci analogous to Nichols *tprE* (*tprG1*) and *tprJ* (*tprG2*) (A), and *tprG/I* hybrid (B) found in the locus analogous to Nichols *tprG* and *tprF*.

The heterogeneity is localized in seven discrete variable regions (V1 to V7) flanked by highly conserved sequences (6). In the Cuniculi A strain of the rabbit treponeme, however, heterogeneity was identified in only a subset of V regions (Fig. 7),

corresponding to *T. pallidum* V1, V2, V4, V6, and V7; V3 is not variable, at least in this isolate of *T. paraluiscuniculi*, and V5 shows very limited sequence diversity. The Cuniculi A V regions differ in their degree of heterogeneity, but diversity is, overall, less impressive than in the human treponeme. As in *T. pallidum*, V6 is the most diverse (eight sequences in 10 clones).

In addition to the differences in the number of V regions between the rabbit and human treponemes, there were 27 additional amino acid differences in the conserved regions compared with the sequence of the Nichols strain. Most were randomly dispersed throughout the conserved regions except for a region of 20 amino acids located immediately upstream of V6. This peptide sequence (SGDPYTHLLTGLNAGVEARV, amino acid positions 367 to 387 of *tprK-C* clones; Fig. 7) differs significantly from the corresponding TprK sequence in the human treponemes and has no homology to any of the seven V regions. Further analysis of this peptide sequence revealed that it matched a highly conserved region of TprL in both the Nichols and Cuniculi A strains and was also present in the carboxyl terminus of the Nichols subfamily I and II antigens.

Antibody and T-cell responses. Pooled sera collected from six *T. paraluiscuniculi*-infected rabbits at nine different time points (day 0 to day 270) were tested by ELISA against recombinant peptides based on the Nichols strain Tpr sequences (Table 2). Sera collected prior to infection showed no specific

TprA-N	A	1	MRLVLGSCIFILLLRGRATVSRHLHASPAVTISGSTRLTWGINLGAKANFVLPVAPLGGATGMVRENPNHRFRHRRHGFRRS
TprA-C		1	MRLVLGSCIFILLLRGRATVSRHLHASPAVTISGSTRLTWGINLGAKANFVLPVAPLGGATGMVRENPNHRFRHRRHGFRRS
TprA-N	A	81	STLFFSLTLCPPKTRSNLHKSSGVYAEILLRNLCCALPLGSLSGEELGELTPTEKQSFVVEATLRFYGAYLTIKKNPTFS
TprA-C		81	STLFFSLTLCPPKTRSNLHKSSGVYAEILLRNLCCALPLGSLSGEELGELTPTEKQSFVVEATLRFYGAYLTIKKNPTFS
			frameshift *
TprA-N	A	161	KNFAKLWPEFITTRYKEADTQYAPGFGDYGGKIGYRVEDVGNSGLGFDGFLSFAASNGDWSTSGTSHSKYGFGSDLISMVQ
TprA-N	A	1	-----MGLVVVTSLW
TprA-C		161	KNFAKLWPEFITTRYKEADTQYAPGFGDYGGKIGYRVEDVGNSGLGFDGFLSFAASNGDWSTSGTSHSKYGFGSDLISLW
TprA-N	A	241	EKQEA V F N C G T R R -----
TprA-N	B	10	YKRM-----KKLFLTVELAGNATLQEGYATLAPTESGAPNNKRASHALLWSVGGRLSIMPAGGRFILATDAGNTYRD
TprA-C		240	YKRM-----KKLFLTVELAGNATLQEGYATLDSRELDGAPNNKRASHALLWSVGGRLSIMPAGGRFILATDAGNTYRD
TprA-N	B	83	TNSARARVVEQALELAEKTYPSLRTVRRIFSWMVQHVDSLGI DALVTAQWRWLSGGVYGATGAASVFGSPFVKSTFYQT
TprA-N		313	TNSARARVVEQALKFAEKTYPSLRTVRRIFSWMVQHVDSLGI DALVTAQWRWLSGGVYGATGAASVFGSPFVNSTFYQT
TprA-N	B	163	DFAAFLRLETRSGDDYTHALHGLNAGIEARVYLPLIGYKSYLDNGGLPPDVVPAQGVAAQQVAMAAARVCLGGLNEMLSATN
TprA-C		393	DFAAFLRLETRSGDDYTHALHGLNAGIEARVYLPLIGYKSYLDNGGLPPDVVPAQGVAAQQVAMIFEAGLRNIFGPFNRVAVN
TprA-N	B	243	FVARAAGLPADAINTGAASIALPIMGNAWVGRI PCYDSMWIIEPRAHIYMATNRFNFGFKGTRNLKGLLFCFYAVELRA
TprA-C		473	FVARAAGLPADAINTGAASIALPIMGNAWVGRI PCYDSMWIIEPRAHIYMATNRFNFGFKGTHNLKGLLFCFYAVELHA
TprA-N	B	323	SPIKPVEFSVRWEQGLLSQDPYMLIEENWVSWP GYTGSLFLGCKITW
TprA-C		553	SPIKHVEFSVRWEQGLLSQDPYMLIEENWVSWP GYTGSLFLGCKITW

FIG. 5. Alignment of TprA homologs in *T. paraluiscuniculi* and *T. pallidum*. TprA-N, *T. pallidum* Nichols strain sequence; TprA-C, *T. paraluiscuniculi* sequence. A, open reading frame A (NH₂-terminal region); B, open reading frame B (central unique and COOH-terminal regions). *, frameshift position in *T. pallidum* TprA (amino acid position 237 of TprA-N, A ORF).

TprL-C	1	MR YAP GF GGY G G K L G Y R A Q D I G G S G I G L D V G F L S F A S N G M W D S T G T A H S K Y G F G A D A T L T Y T H H R A E R I K M E L A G N A T L E
TprL-N	1	MR YAP GF GGY G G K L G Y R A Q D I G G S G I G L D V G F L S F A S N G M W D S G - T A H S K Y G F G A D A T L T Y T H H R A E R I K M E L A G N A T L E
TprL-C	81	P K Y T T G T E Q G R K M D Q K D S L L W S A G G R L T L T P G Y G F R L V L A L D V G N I H R S D E N M E K T M R W E E K A G V A V A K A V A E L Q Q C I L Q
TprL-N	80	P C Y T T G T E Q G K M M E Q K N R L L W S A G G R L T L T P G Y G F R L V L A L D V G N I H R S D A D I G K T V N V Q A K A A E A V S A A V T E F W A Q V A Q
TprL-C	161	I V R N G G V V D F F V K K V G G A S L I E Q V G L V V S M L G G K L I N V L S S A A Q Q V A R V A E N L T K K F A E L L E R P D P A I A L V K F F A W L D R L
TprL-N	160	I M A N G G V G E F F V K K V R G A A L I A Q V A L V V S H L E G K L S N L L C S T L G - L G A V V N Q L T Q G F A E L L K K P D P A I A L V I F F A W L H R L
TprL-C	241	R V H M L G A D A L L C M Q W K W L S S G A Y F A T A G A N M F G K R V F S R Q F A D D Y L D C A A F L K L E T K S G D P Y T H L L T G L N A G V E A R L Y I P
TprL-N	239	H V H E L G A D A L L S M Q W K W L S S G A Y F A T A G A N M F G K R V F S R Q L T - D Y L D C A A F L K L E T K S G D P Y T H L L T G L N A G V E A R L Y I P
TprL-C	321	F T Y T S Y L D N G G I A Y N G R T A G G H I N L P V V G K T W L S Y Q I A L G S H A W L K P Y A V V Y G T T N R F N T D M A S N L L C E K A M Q Y H V G F T V
TprL-N	318	F T Y T S Y V D N G G I D Y K K T T M R G P I N L P V V G K T W L S Y Q I A L G S H A W L K P Y A V V Y G T T N R F N T D K A M N L L R E K A M Q Y H V G F T V
TprL-C	401	S P I E K V E F D A R W E Q G R L A T S P Y M L I T E D I S S D K H F G T F V C G L K I A W
TprL-N	398	S P I E K V E F D A R W E Q G R L A T S P Y M L I T E D I S S D K H F G T F V C G L K I A W

FIG. 6. Alignment of TprL homologues in *T. paraluiscuniculi* and *T. pallidum* subsp. *pallidum*. TprL-C, *T. paraluiscuniculi* sequence; TprL-N, *T. pallidum* Nichols strain sequence.

reactivity against any of the recombinant antigens tested. ELISA background absorbance readings ranged from 0.001 to 0.03 units. Immune sera raised against each of the recombinants were used as positive controls and to assess binding of the antigen to the plate well (data not shown). All sera tested (pre- and postinfection and positive-control sera) were not reactive against the unrelated SA85 1.1 *Trypanosoma cruzi* recombinant protein (24) used as a control antigen to assess cross-reactivity (data not shown).

Antibody responses to Cuniculi A strain subfamily I members (TprC and TprD) were determined with five recombinant peptides (5'-subfamily I, TprF, TprD2-central, and 3'-subfamily I A/B; Fig. 8). TprF and the 5'-subfamily I recombinant share a sequence of 267 amino acids (the conserved amino terminus of subfamily I). The strongest reactivity peak against these two recombinants was detected at day 30, decreasing gradually over time, with virtually no detectable reactivity by day 190. We interpret this antibody reactivity in infected rabbits as the immune response against the amino terminus of the Cuniculi A TprD2-like peptides due to sequence conservation in this region in all members of subfamily I. Although the Cuniculi A *tprC* and *tprD* loci contain frameshifts which cause premature stops, it is still possible that the coding region upstream of the frameshift could be translated. The TprD2-central and the 3'-subfamily I A/B recombinant peptides were not recognized by the antisera (Fig. 8), consistent with the hypothetical partial translation of TprC and TprD.

The TprG-central and 3'-subfamily II recombinant peptides were used to detect antibody responses against Cuniculi A TprG1 and TprG2. These two identical homologs encode hybrid proteins which contain the following Nichols fragments: the amino terminus of subfamily II (containing a frameshift), the unique central region of TprG, and the carboxyl terminus of TprJ (Fig. 2 and Fig. 4A). The antisera showed no reactivity (Fig. 8), most likely because these recombinants represent proteins that are not translated due to the frameshift in the Cuniculi A coding sequences. No recombinant peptide was available to investigate the antibody response against the NH₂-terminal portion of the subfamily II Tprs. Antisera were also

tested against two antigens (TprE-central and TprJ-central) representing the central regions of Nichols subfamily II members TprE and TprJ. These sequences have a very low degree of homology with any of the Cuniculi A antigens described (Table 2), but they were tested to rule out expression of unidentified Tpr antigens with significant homology to these two Tprs elsewhere in the chromosome. No significant reactivity against them was detected (Fig. 8). Antibodies against the Cuniculi A TprG/I hybrid were investigated with the TprI-central peptide, corresponding to the hybrid's central unique region, but no reactivity was found (Fig. 8). As in subfamilies I and II, this suggests no translation due to the frameshift upstream of this region.

Five peptides (TprA N-terminal, TprA C-terminal, TprB-central, TprH-central, and TprK-central) were tested to assess reactivity against subfamily III members. Antisera reacted against TprK, with the highest and strongest reactivity at day 30 postinfection (Fig. 7). No significant antibody reactivity was detectable against TprA N-terminal, TprA C-terminal, TprB-central, and TprH-central.

T-cell responses against the *T. paraluiscuniculi* Tprs were assessed with the same recombinants used for the ELISA tests (Table 2) plus 40 additional peptides (39 synthetic and 1 recombinant) spanning the entire Nichols TprK sequence (unpublished data). Concanavalin A-stimulated splenic lymphocytes from infected and uninfected rabbits showed strong proliferative responses, while no significant proliferation was detected in the absence of antigen (data not shown). Splenocytes from *T. paraluiscuniculi*-infected rabbits had significant [³H]thymidine incorporation when stimulated with *T. pallidum* sonicate and, in contrast, splenocytes from uninfected rabbits failed to proliferate in the presence of *T. pallidum* sonicates (data not shown). Of all the recombinant peptides listed in Table 2, only the TprK-central recombinant elicited a significant response in infected rabbits (Fig. 9A); no response was found to any other recombinant peptides (data not shown). Proliferation was seen in response to synthetic peptides 1, 3, 10, and 15 and recombinant peptide 40, all of which carry

Nichols TprK translational start site

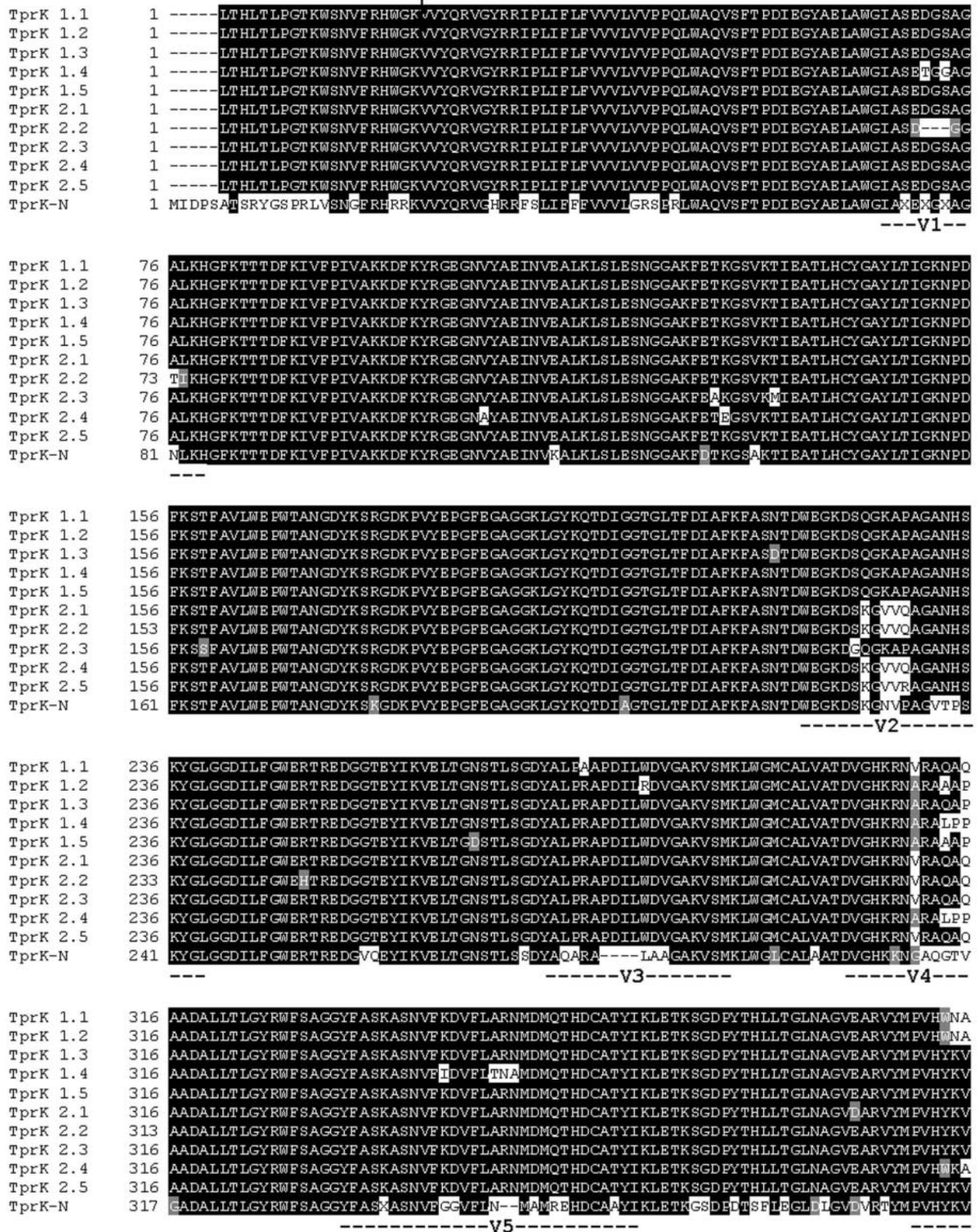


FIG. 7. Alignment of TprK deduced amino acid sequences in *T. paraluiscuniculi* and *T. pallidum* Nichols strain. TprK-N, *T. pallidum* Nichols strain sequence. Amino acid sequences upstream of the Nichols TprK translational start site are included for comparison (4, 15). TprK 1.1 to 1.5 and TprK 2.1 to 2.5, *T. paraluiscuniculi* sequences from two independent PCRs; V1 to V7, TprK variable regions (6).



FIG. 7—Continued.

highly conserved TprK regions. None of the other peptides induced significant T-cell responses (Fig. 9B).

The *tpr* gene family in *T. paraluiscuniculi* is composed of at least 10 paralogs; six of them are single-copy genes, and two have identical alleles in two different loci. These characteristics are summarized in Table 3, along with prediction of cellular location, molecular weight, and presence of a signal peptide.

Sequence analysis predicts that half of these genes are non-functional due to the presence of frameshifts and premature stop codons; only the subfamily III ORFs are presumably fully functional. Antibody and T-cell assays show, however, that only TprK is recognized by the humoral and cellular immune response, suggesting that the other subfamily III members may not be expressed. The diversity in Cuniculi A TprK resembles that observed in *T. pallidum* and suggests the presence of an antigenic variation system similar to the one described for the human treponeme (6, 7).

DISCUSSION

The Tpr antigen families of *T. pallidum* and *T. paraluiscuniculi*, pathogenic spirochetes which cause human and rabbit venereal syphilis, respectively, were the focus of this comparative study. Both spirochetes cause persistent infections, are transmitted from host to host by a similar route, and are indistinguishable in morphology and the immunopathological changes induced. However, the human venereal syphilis spirochete has the ability to infect both humans and rabbits, while the rabbit treponeme has a more restricted host specificity, being unable to cause active infection in humans (14).

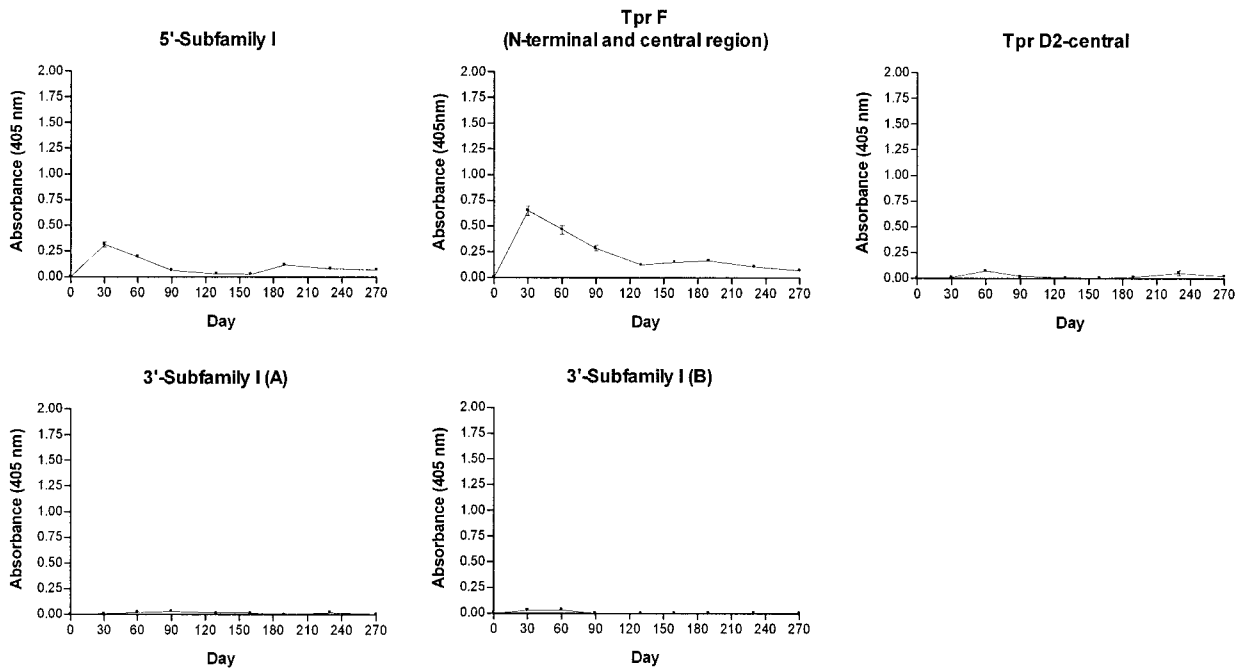
Morphological, genetic, and immunological analyses of *T. paraluiscuniculi* and the other pathogenic treponemes indicate that these organisms are highly related. It has been postulated that the pathogenic treponemes arose originally from free-living treponemes in mud (29). These organisms came to be

carried commensally by animals and humans and then developed the ability to cause disease as natural selection ensured the optimal survival of mutants best suited for transmission from host to host in the prevailing environment (31). It is most likely that the rabbit spirochete and the other treponemes evolved with mechanisms similar to those of other organisms, by acquisition or loss of new traits resulting in differences in host specificity and mechanisms of pathogenesis.

The *tpr* gene family likely arose by gene duplication events in the distant past. Duplication of an ancestor *tpr* gene during evolution may have generated this paralogue family, whose members then underwent further differential evolution. Some might have been randomly silenced, while others might have undergone neofunctionalization (one copy acquires a novel, beneficial function and becomes preserved by natural selection, while the other copy retains its original function) (20, 22) or subfunctionalization (complementary loss of function of both copies to the level of the single-copy ancestral gene, resulting in the preservation of both loci) (21).

All the subfamily I (*tprC*, *tprD*, *tprF*, and *tprI*) and II (*tprE*, *tprG*, and *tprJ*) *tprs* in the human syphilis treponeme Nichols strain are thought to be transcribed and translated (4, 27; unpublished data) and, except for *tprF*, do not contain frameshifts and early termination. In contrast to *T. pallidum*, all subfamily I (*tprC* and *tprD*), II (*tprG1* and *tprG2*), and the I/II hybrid (*tprG/I*) ORFs identified in *T. paraluiscuniculi* are predicted to encode truncated proteins due to frameshifts caused by a single extra guanosine in their coding sequences. This predicts that these genes are not essential for survival in the rabbit. Sequences obtained from two independent PCRs and from shorter amplicons containing the frameshift regions (data not shown), obtained with primers internal to the ones used for amplification of the whole ORFs, ruled out the possibility that the extra G nucleotide was due to PCR error. In addition, it is highly unlikely that PCR errors would be introduced at the

Subfamily I



Subfamily II

Tpr G/I hybrid

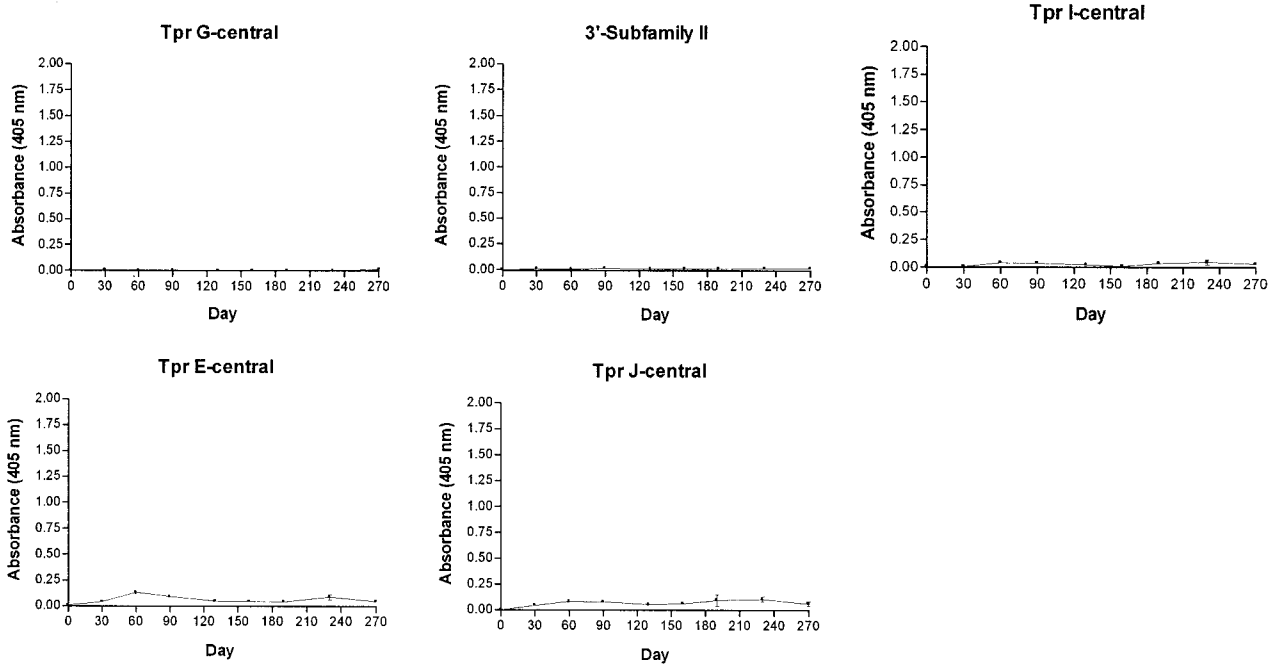


FIG. 8. Antibody responses of *T. paraluiscuniculi*-infected rabbits to Tpr recombinant peptides through day 270 postinfection as measured by ELISA. Peptides were designed based on the Nichols strain Tpr sequences. The reported values (mean OD₄₀₅ ± standard error of the mean) represent three replicates of pooled sera from six Cuniculi A strain-infected rabbits.

Subfamily III

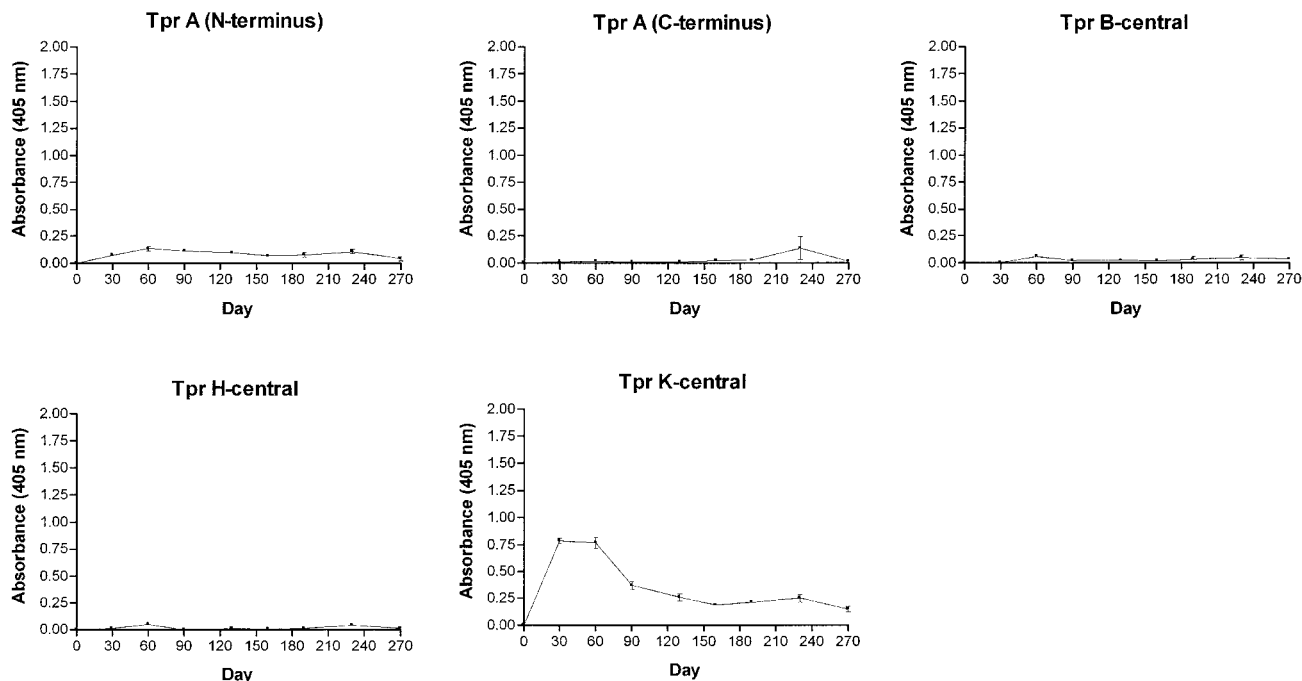


FIG. 8—Continued.

same nucleotide position for *tprC* and *tprD* and, for the *tprG1* and *tprG2* alleles, in two independent PCRs for each template. It is also possible, however, that the frameshifts might be corrected in a small proportion of mRNAs during transcription or that ribosomal slippage occurs during translation, permitting translation of a complete product.

The *T. paraluisuniculi* *tprD2*-like sequences found in the *tprC* and *tprD* loci are virtually identical to the *tprD2* allele present in other *T. pallidum* isolates. Approximately 50% of human syphilis isolates contain the *tprD2* gene; some humans with venereal syphilis make antibodies to a TprD2-specific peptide (8). *tprD2* is also found in *T. pallidum* subsp. *pertenue* and *T. pallidum* subsp. *endemicum*. The TprD2 antigen is predicted to be an outer membrane antigen and may provide functional diversity to individual isolates. It is notable that both *tprD2*-like alleles in the rabbit treponeme contain authentic frameshifts introducing early terminations and generating a short predicted peptide sequence of only 196 amino acids (instead of 597). The frameshift is due to an extra G located at exactly the same nucleotide position in both loci, suggesting that the frameshifts are not artifactual and that these two alleles are nonfunctional. In contrast, the *T. pallidum* *tprD2* homologue has been retained intact during evolution, and in the presence of selective advantage, it might have undergone neofunctionalization. This could provide advantages to particular *T. pallidum* isolates, such as adaptation to specific tissues in the human host.

Due to the presence of frameshifts and premature stops in the subfamily I Tprs, we reasoned that only the NH₂-terminal portion of these genes is likely expressed and that the central and COOH-terminal regions are not translated. This is supported by our ELISA findings, where antibody reactivity was seen against the conserved subfamily I amino terminus and no reactivity was detected against the central region (TprD2-central) or the carboxyl terminus (3'-subfamily I peptides A and B). Alternative explanations for the lack of reactivity against the central and COOH-terminal regions are that these regions are not the target of the humoral antibody responses and that they are expressed at a very low level during infection. However, the recent demonstration that human syphilitic sera react against a 216-amino-acid peptide of the subfamily I Tprs carboxyl terminus (V. Sambri et al., unpublished data) supports the idea that the absence of reactivity to this particular region in the Cuniculi A strain is due not to lack of antigenicity but to lack of translation. This hypothesis is equally supported by the absence of detectable reactivity against the central region and carboxyl terminus of Cuniculi A subfamily II members (TprG1 and TprG2) and the TprG/I hybrid, with these regions being coded by sequences downstream of the frameshift position in their respective genes. No recombinant antigens were available to test reactivity against the amino terminus of these antigens due to difficulties in expressing this particular peptide region.

Based on the analysis of the predicted protein sequences, it appears that only the subfamily III Tpr proteins may be func-

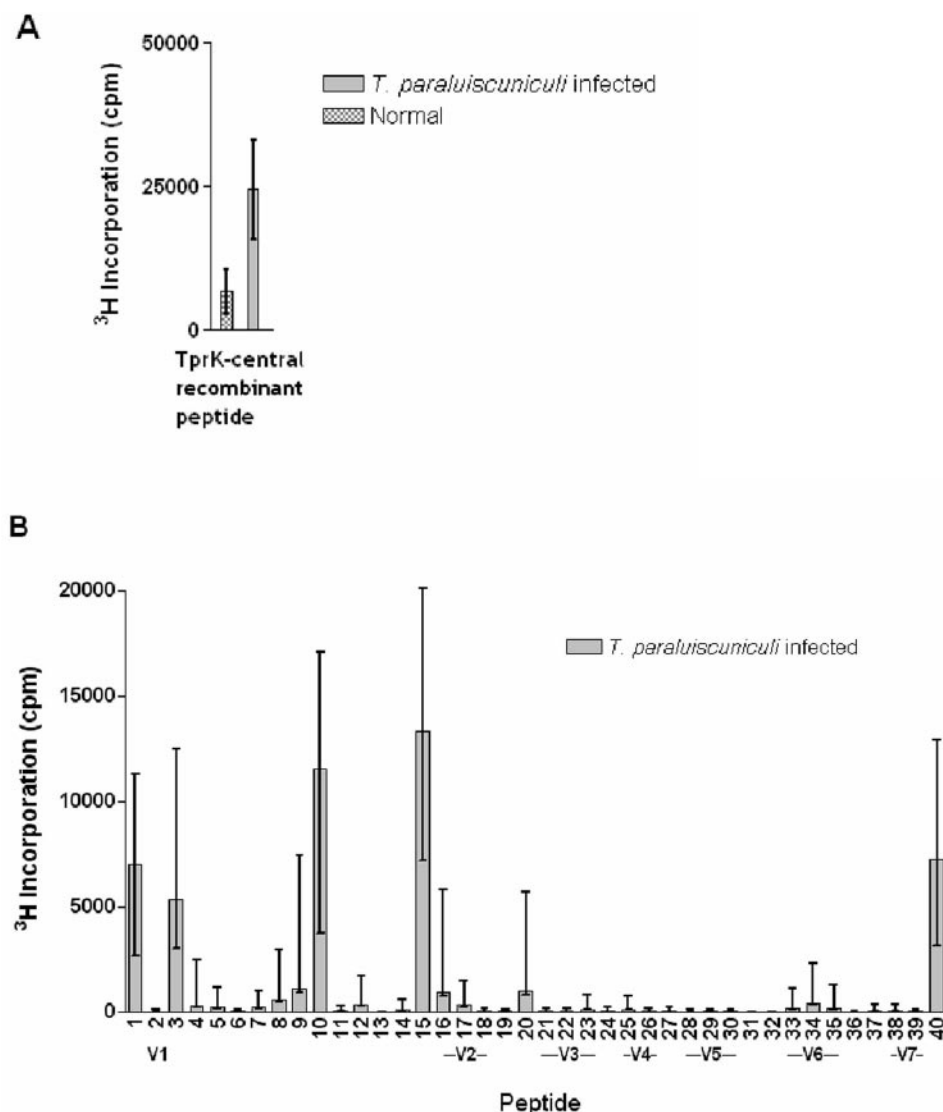


FIG. 9. T-cell epitopes. Splenocytes from five rabbits infected for 270 days with *T. paraluiscuniculi* Cuniculi A strain were tested for their ability to proliferate when stimulated with recombinant TprK-central peptide (A) and synthetic peptides (B). All peptides were designed according to the Nichols strain Tpr sequences. A. TprK-central (315 amino acids, Table 2) encompasses the sequences of peptides 2 to 30 and partially those of peptides 1 and 31. The concentration of antigen was 10 μ g/ml. B. Among the peptides encompassing sequences in the conserved regions of TprK, T-cell response was confined to peptides 1, 3, 10, 15, and 40 during *T. paraluiscuniculi* experimental infection. V1 to V7 indicate peptide amino acid sequences found in the variable regions (unpublished data).

tional molecules in *T. paraluiscuniculi*. There is a very high degree of conservation between the *T. paraluiscuniculi* and *T. pallidum* *tprB* and *tprH* genes, which are virtually identical. In contrast, there are significant sequence differences in their *tprA*, *tprK*, and *tprL* genes. *tprA* is an intact ORF in the rabbit treponeme but not in *T. pallidum* Nichols strain, where this ORF has an authentic frameshift due to the presence of only three CT repeats instead of four. In absence of full-length functional *tprC*, *tprD*, *tprF*, *tprI*, *tprG*, *tprJ*, and *tprE* genes, one could assume that *tprA* and the other subfamily III members having intact full-length coding sequences are playing an important role in *T. paraluiscuniculi* biology. Although this assumption may seem obvious from sequence analysis, ELISA

antibody reactivity and proliferation assays do not entirely support this hypothesis.

Overall, antibody responsiveness to subfamily III Tpr antigens in *T. pallidum* isolates has been shown to vary in specificity and temporal pattern of reactivity elicited against individual Tpr proteins during infection (18). Of particular interest is the Nichols strain, in which the expression level of most subfamily III Tprs (except for TprK) seems to be lower than in other *T. pallidum* isolates. It has been suggested that this may reflect a high degree of adaptation to the rabbit, which by analogy could be extrapolated to *T. paraluiscuniculi* as well. The lack of antibody specificities during *T. paraluiscuniculi* infection is similar to that seen against subfamily III members

in the Nichols strain, in which TprK is the only apparent target of the immune response. T-cell proliferation data confirm these observations, with TprK being the only target of the cellular immune response. As in *T. pallidum* (24), Cuniculi A T-cell epitopes were found in the TprK conserved regions, with the same peptides being recognized in both infections. The lack of antibody and T-cell responses to the other subfamily III Tprs (in the presence of intact ORFs) may reflect different expression patterns (very low levels of expression throughout infection) or lack of antigenicity of these proteins, or may indicate that these ORFs are actually be pseudogenes.

The *tprK* locus is of particular interest. It has been shown in *T. pallidum* that the TprK antigen contains seven variable regions targeted by the antibody response of the host and that it undergoes antigenic variation during infection with donor sites located in a \approx 4-kb region flanking the *tprD* gene (24, 7). As in *T. pallidum*, Cuniculi A TprK is the target of an early and strong immune response. The heterogeneity in the V regions of the *tprK* alleles in the Cuniculi A isolate suggests that its TprK may undergo antigenic variation too. Interestingly, a significant difference is the presence of limited diversity in the V regions in the rabbit treponeme, with the observed heterogeneity not being as impressive as in the human isolates. It has been postulated that the extensive diversity in the TprK antigen may represent mechanisms for immune evasion and adaptation to a changing environment in the host during infection. In this context, the overall lower diversity in the Cuniculi A TprK antigen might imply that *T. paraluisuniculi* uses a smaller subset of TprK variants to achieve successful adaptation in its host.

So far, we have been able to obtain only a fragment of about 600 nucleotides from the corresponding Cuniculi A donor regions flanking the *tprD* gene by PCR amplification. We do not know at this time whether this is due to lack of binding sites for the primers, fragmentation, and different localization of the donor sites in the chromosome, or smaller donor regions in *T. paraluisuniculi* than in *T. pallidum*.

The successful amplification of the *tpr* ORFs in *T. paraluisuniculi* with primers based on the Nichols genome sequence of the flanking regions for each locus suggests sequence conservation at the primer binding sites and similar organizations of the ORFs (*tprs* and flanking ORFs) in the amplified regions of these two species. We sequenced a large number of *tpr* ORFs from several *T. pallidum* isolates and observed variations in sequence composition in the coding regions but not in their chromosomal locations, supporting earlier morphological and immunopathological observations that these are highly related organisms. However, we cannot completely rule out the presence of additional paralogues in the Cuniculi A chromosome. Alternative approaches, such as Southern blot analysis or the construction of genomic, subgenomic, and lambda libraries, have already been considered. Although technically feasible, *T. paraluisuniculi* grows very slowly with very low bacterial yields, making it impractical to collect Percoll-purified treponemes (free of most contaminating rabbit DNA) in sufficient numbers to create these libraries. It is more likely that other approaches that require minimal amounts of DNA (such as whole-genome long-PCR fingerprinting) might yield more information without the need for large amounts of treponemal DNA.

It is intriguing to find five apparently nonfunctional *tpr* genes in *T. paraluisuniculi* due to frameshifts and premature stops (*tprC*, *tprD*, *tprG1*, *tprG2*, and the hybrid *tprG/I*). It is likely that these genes were silenced during evolution after gene duplication generated the *tpr* family in an ancestor treponeme. One hypothesis is that the human and rabbit treponemes arose from a common predecessor containing a set of fully functional *tpr* genes. Later on, during adaptation to the genetic background of the rabbit, the subfamily I and II genes were then silenced by single-base insertions. However, to explain the presence of identical alleles in the *tprC* and *tprD* loci as well as in the *tprG1* and *tprG2* loci containing frameshifts at exactly the same positions, we need to assume that the original set of genes were composed of a smaller number of single-copy genes, some of which underwent further duplication after the frameshifts were introduced. The lack of functionality of these genes suggests that they are not essential for survival in the rabbit but may represent pathogenicity factors in *T. pallidum*. As mentioned above, we have not completely ruled out the possibility of other *tpr* genes elsewhere in the Cuniculi A chromosome, and ongoing studies in our laboratory will determine whether second functional copies of these genes are present in this strain and, also, whether other *T. paraluisuniculi* strains have similar sets of genes and similar patterns of expression.

Besides the most obvious differences between the *T. pallidum* and *T. paraluisuniculi* homologues presented here, small numbers of single synonymous and nonsynonymous nucleotide mutations can also be identified in the *tpr* sequences. The amino acid changes due to single-nucleotide mutations that might influence protein function can be observed throughout some Tpr sequences, suggesting selective evolutionary divergence. Nevertheless, silent mutations are also known to influence protein function by mediating in vivo protein folding changes or regulating gene transcription (9, 10). Although the sequence organization of and host immune responses to the Tpr antigens are being unraveled, the function of these proteins and the role in antigenicity or protein structure of the observed diversity due to single-nucleotide changes or more extensive sequence modifications still remain unclear.

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