Sequence Diversity of *Treponema pallidum* subsp. *pallidum* *tprK* in Human Syphilis Lesions and Rabbit-Propagated Isolates

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Received 19 May 2003/Accepted 11 August 2003

The *tprK* gene of *Treponema pallidum* subsp. *pallidum*, the causative agent of venereal syphilis, belongs to a 12-member gene family and encodes a protein with a predicted cleavable signal sequence and predicted transmembrane domains. Except for the Nichols type strain, all rabbit-propagated isolates of *T. pallidum* examined thus far are comprised of mixed populations of organisms with heterogeneous *tprK* sequences. We show that *tprK* sequences in treponemes obtained directly from syphilis patients are also heterogeneous. Clustering analysis demonstrates that primary chancre *tprK* sequences are more likely to cluster within a sample than among samples and that tighter clustering is seen within chancre samples than within rabbit-propagated isolates. Closer analysis of *tprK* sequences from a rabbit-propagated isolate reveals that individual variable regions have different levels of diversity, suggesting that variable regions may have different intrinsic rates of sequence change or may be under different levels of selection. Most variable regions show increased sequence diversity upon passage. We speculate that the diversification of *tprK* during infection allows organisms to evade the host immune response, contributing to reinfection and persistent infection.

The spirochete *Treponema pallidum* subsp. *pallidum* causes multistage disease and establishes persistent infection in the absence of antibiotic therapy. Although robust humoral and cellular immune responses are able to clear most treponemes from primary and secondary syphilis lesions, some treponemes survive to establish lifelong persistent infection. The mechanism by which these organisms evade the immune response to persist for decades is not known.

Like gram-negative bacteria, *T. pallidum* has an inner and an outer membrane, but freeze-fracture electron microscopy has shown that, compared to *Escherichia coli*, the outer membrane of *T. pallidum* is relatively devoid of integral membrane proteins (19, 25). The extreme fragility of *T. pallidum* to physical manipulation has thus far impeded attempts to ascertain the identity of these rare outer membrane proteins (5). The *tpr* family of genes, some of which have homology to the major surface protein genes of *Treponema denticola*, has been identified in *T. pallidum* (3, 7, 21). Cleavable signal sequence and transmembrane domains are predicted for several *Tpr*s (3), making them candidates for outer membrane exposure. Examination of sequences from various clinical isolates demonstrates that one of the *Tpr*s, *TprK*, is heterogeneous in seven discrete variable (V) regions that are separated by conserved sequences (4, 20). Differences in the V regions are due to base pair changes, as well as insertions and deletions that preserve the coding frame of *tprK*. Hazlett et al. (8) showed that the *tprK* translational start is downstream of the start site predicted in the *T. pallidum* genome sequence (7), which supports the presence of a cleavable signal sequence upstream of the *TprK* V

regions. There is a single *tprK* locus in the genome of the laboratory strain Nichols and Street strain 14 (20), and our laboratory has been able to isolate populations of *T. pallidum* that are clonal in *tprK* (unpublished results). This suggests that heterogeneous *T. pallidum* isolates are comprised of populations of organisms that each carry a single, unique *tprK* sequence.

A model has been proposed for a segmented gene conversion-like mechanism of variation of *tprK* (A. Centurion-Lara, R. E. LaFond, K. Hevner, C. Godornes, B. J. Molini, and S. A. Lukehart, unpublished data). Each *tprK* V region is flanked by a pair of unique terminal direct repeats, and internal repeat sequences are also found within each V region. Whole or partial fragments of *tprK* V region sequences, including terminal and/or internal repeat sequences, are found in donor regions adjacent to the *tprD* gene. In this model, DNA segments are copied from donor sites and inserted into the *tprK* expression locus V regions, creating new sequences in the insertion site. Donor cassettes can introduce single- or multiple-base-pair changes, insertions, or deletions found among *tprK* V regions. This model allows for the generation of the wide array of *tprK* variants found in *T. pallidum* isolates.

We demonstrate here that *tprK* diversity is present during syphilis infection in individuals and accumulates upon rabbit passage of *T. pallidum*. Clustering analysis shows that *tprK* sequences from rabbit-propagated isolates are more closely related within than among isolates. Sequences derived directly from syphilis patients are diverse but cluster more tightly within infected individuals than do rabbit-propagated isolates. Individual V regions from a rabbit-propagated isolate display differing levels of heterogeneity, and we show that a low-heterogeneity V region accumulates diversity upon serial passage.

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MATERIALS AND METHODS

Collection and isolation of *T. pallidum* samples. Swabs were obtained from the primary chancres of three patients with early syphilis (MD504, MD530, and

MD55) at the Sexually Transmitted Disease Clinic at Johns Hopkins University, Baltimore, Md. *T. pallidum* was detected by dark-field microscopy in the chancres of all three patients, and MD504 and MD530 had reactive serum Reactive Plasma Reagin tests for syphilis. The present study was approved by the Human Subjects Divisions of Johns Hopkins University and the University of Washington. Swabs were stored in 500 μ l of 1 $\times$ lysis buffer (10 mM Tris, pH 8; 0.1 M EDTA, pH 8; 0.5% sodium dodecyl sulfate). *T. pallidum* Sea 81-4 was isolated by rabbit inoculation of material collected from the primary chancre of a patient attending the Seattle-King County STD Clinic at Harborview Medical Center, and the isolate was propagated by serial intratesticular passage through New Zealand White rabbits (R&R Rabbitry, Kenwood, Wash.) as previously described (10). *T. pallidum* Sea 81-3, Sea 83-1, and Sea 84-2 were isolated by rabbit inoculation of the cerebrospinal fluid from three patients with neurosyphilis (11), and serially passaged in New Zealand White rabbits 4, 6, and 10 times, respectively. The respective committees at the University of Washington approved all human subject and animal protocols. Treponemes were harvested from infected rabbit testes and enumerated by dark-field microscopy. For DNA sequencing, bacteria were pelleted at 12,000 $\times$ g for 30 min and resuspended in 1 ml of 1 $\times$ lysis buffer.

Treponemal DNA extraction and PCR amplification of *tprK*. DNA was extracted from bacterial pellets and swab samples by using the QIAamp DNA Mini kit (Qiagen, Inc., Valencia, Calif.), taking careful precautions to avoid DNA cross-contamination between isolates. For Sea 81-4, DNA was extracted from a known number of treponemes, and an aliquot of DNA equivalent to 100 organisms was used for amplification of *tprK*. DNA was amplified in this manner from serial passages of *T. pallidum* Sea 81-4: 4 (low-passage), 12 (medium-passage), or 21 (high-passage) rounds after isolation from the patient. DNA was also amplified from rabbit-passaged *T. pallidum* isolates Sea 81-3 (passaged 4 times), Sea 83-1 (passaged 6 times), and Sea 84-2 (passaged 10 times). The primers used for amplification of DNA from isolates encompass the seven V regions of *tprK* (sense, 5'-ATATTGAAGGCTATGCGGAGCTG; antisense, 5'-TACCCACACCTCGTAATACCC). Sea 81-4 DNA was amplified with *Taq* DNA polymerase (Promega Corp., Madison, Wis.) by using PCR conditions as described elsewhere (4), except that the annealing temperature was 60°C and the number of cycles was 45. PCR amplification of the three cerebrospinal fluid isolates was performed with 1 μ M concentrations of the above primers in a 100- μ l reaction containing a 200 μ M concentration of deoxynucleoside triphosphates (Promega), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 0.1 mg of bovine serum albumin/ml, 2.5 U of *Pfu* Turbo Hotstart DNA polymerase (Stratagene, La Jolla, Calif.), and 5 μ l of purified DNA. Cycling conditions were as described elsewhere (4) except that the annealing temperature was 60°C. Primers used for amplifying primary chancre DNA encompass the open reading frame of *tprK* (sense, 5'-GTGGTGTATCAGCGGGTAGG; antisense, 5'-GACATGCCCTACGAATTG). PCR conditions were as described above for Sea 81-4.

Clustering analysis of *tprK* sequences. *tprK* PCR products were cloned and sequenced as described elsewhere (4). Sequences were deposited to GenBank under accession numbers (AY346012 to AY346089). DNA and translated amino acid sequences of *tprK* containing all seven V regions (corresponding to bp 182 to 1380 of the *T. pallidum* genome *tprK* sequence) were aligned by using CLUSTAL W (European Molecular Biology Laboratory, European Bioinformatics Institute [http://www.ebi.ac.uk/clustalw/]) (24), and alignments were adjusted manually to correct misalignments or gaps introduced by the program. DNA sequence alignments were subjected to neighbor-joining and parsimony analysis by using PHYLIP (Phylogeny Inference Package, version 3.57c; Department of Genetics, University of Washington [http://evolution.genetics.washington.edu/phylip.html]). The trees were subjected to 100 random bootstrap resamplings, and bootstrap confidence levels were determined; values of >70 are considered to be significant (6).

RESULTS

Comparative analysis of diverse *tprK* sequences from patient samples and rabbit-propagated isolates. In the present study, we sought to analyze *tprK* sequences from treponemes obtained directly from the primary chancres of three patients, and compare them to *tprK* sequences from three rabbit-propagated isolates. The *tprK* gene was either amplified directly from swabs of primary chancres of three patients with early syphilis—MD504, MD530, and MD55—or was amplified from

the harvested treponemes of the rabbit-propagated isolates Sea 81-3, Sea 83-1, and Sea 84-2. The amplicons were then cloned into plasmids and sequenced. Sequencing of 14 plasmid clones from MD504 and 9 plasmid clones from MD55 yielded eight and seven different *tprK* sequences, respectively. Five different sequences were found in the 13 plasmid clones analyzed from MD530. Sequences that are heterogeneous in at least one of the seven V regions in *tprK* within each sample are considered different alleles; each sequence is identified by a lowercase letter, and amino acid alignments of the V regions of the unique sequences from each patient sample are shown in Fig. 1A (constant regions are 99.7% identical at the DNA level and are therefore not shown). The N- and C-terminal ends of the V regions in Fig. 1 correspond to the terminal base pair repeats described above. Some V regions are shared within the plasmid clones sequenced from an individual patient, and several are shared between two patients. For example, the V1 sequence GIASDGGAIKH is found in all MD504 plasmid clones, as well as in plasmid clone "a" of MD55, and all but one of the MD55 and MD530 clones share the same V5 sequence. In addition, the V2 sequence in MD504 plasmid clone "h" is found in five MD530 clones, and the V7 sequence YGGT NKKNDAAATKWKAIEYCGYYE is shared by three plasmid clones from MD504 and four plasmid clones from MD55. In contrast to sequences from patient lesions, each plasmid clone from rabbit-propagated isolates yielded a different *tprK* sequence. As in patient-derived samples, heterogeneity in *tprK* is found only in the V regions (Fig. 1B). Although there are many examples of V region sequences that are shared within an isolate (i.e., the V1 sequence GIASDGGAIKH in Sea 81-3), only the V4 sequence TDVGRKKDGAQGTGAD is shared between the Sea 81-3 and Sea 84-2 isolates.

To further explore *tprK* heterogeneity, we determined the number of different sequences for each of the V regions (Table 1). With the exception of V4, there are more different V region sequences in rabbit-propagated isolates than in the patient-derived samples. The rabbit-propagated isolates have a considerably wider range of sequence length in V3 and V6, although V7 has a wider range of length in patient-derived treponemes.

Clustering analysis of *tprK*. In the alignments in Fig. 1, some V regions do not show heterogeneity within a patient sample or isolate. To determine the relatedness within and among *tprK* sequences derived from different sources, we performed clustering analysis by using the neighbor-joining method. The *tprK* sequences from MD504, MD530, and MD55 fall into three tight clusters that correspond to the individual patient samples (Fig. 2A). Bootstrap analysis shows that these identical cluster groups occur in all 100 resampled trees. This indicates that *tprK* sequences are more related within treponemes from an individual patient than among patients.

To test the hypothesis that heterogeneity in *tprK* accumulates during rabbit passage, we compared the *tprK* sequences obtained directly from patient specimens to those from three rabbit-propagated isolates. Clustering analysis of seven, eight, and five different *tprK* sequences, respectively, from *T. pallidum* Sea 81-3, Sea 83-1, and Sea 84-2 are shown in Fig. 2B. As in the primary chancre samples, the sequences generally cluster by isolate, but the genetic distance among the sequences within a rabbit-propagated isolate is greater than seen in the

FIG. 1. Amino acid alignments of the variable (V) regions of *tprK* from directly sequenced patient samples (A) and rabbit-propagated isolates (B). Shaded areas indicate sequence identity; dashes indicate gaps in the alignment. The translational start shown here was identified by Hazlett et al. (8) as different from that in the genome sequence. Conserved regions are identical to those previously published (4, 20); the number of conserved amino acids not shown is indicated.

chancre samples. Two of the isolates have sequences clustering at the end of major branches; only the branch containing sequences from Sea 81-3, which was passaged the fewest number of times, has a bootstrap number higher than the minimum

confidence level of 70. The Sea 84-2 isolate, which underwent the highest number of rabbit passages, does not cluster at the end of a single major branch. This analysis shows that *tprK* sequences from rabbit-propagated isolates are more diverse

FIG. 1—Continued.

than those from directly sequenced patient samples, suggesting that propagation of isolates gives organisms the opportunity to accumulate diversity in *tprK*.

Clustering analysis by using the parsimony method for the trees in Fig. 2 yields bootstrap numbers (data not shown) similar to those resulting from the neighbor-joining method.

Because the differences in *tprK* are due to insertions and deletions of nucleotide sequences of variable lengths in addition to apparent base pair changes, we also performed neighbor-joining and parsimony analysis on sequence alignments with the gaps removed. These analyses yielded results similar to those shown in Fig. 2 (data not shown).

TABLE 1. Analysis of *TprK* V regions from patient samples and rabbit-propagated isolates

V region	No. of different V region sequences in patient sample(s):				Length of V region (aa) ^a in patient samples	No. of different V region sequences in rabbit-propagated isolate(s):				Length of V region (aa) in rabbit-propagated isolates
	MD504 (n = 8)	MD530 (n = 5)	MD55 (n = 7)	Combined (n = 20)		Sea 81-3 (n = 7)	Sea 83-1 (n = 8)	Sea 84-2 (n = 5)	Combined (n = 20)	
V1	1	1	2	3	11–15	2	4	1	7	11–14
V2	3	1	2	5	20–22	6	3	2	11	20–22
V3	1	2	3	6	19–23	3	4	3	10	15–27
V4	3	2	1	6	17–20	2	2	2	5	17–20
V5	3	1	2	5	28	6	5	4	15	28–30
V6	4	4	3	11	15–21	7	5	5	17	13–27
V7	4	3	2	8	24–44	2	5	5	12	23–27

^a aa, amino acid(s).

Accumulation of diversity in *T. pallidum* Sea 81-4 *tprK*. Our analyses of *tprK* have revealed that individual V regions have different degrees of diversity within and among *T. pallidum* isolates (4). To investigate this phenomenon more closely, we examined the unique sequences for each V region in the rabbit-propagated Sea 81-4 isolate. To investigate the possibility that a given V region sequence might change with repeated passage, we sequenced *tprK* from Sea 81-4 organisms harvested after 4 (low-passage), 12 (medium-passage), or 21 (high-passage) rounds of propagation in rabbits.

For each passage level, genomic DNA equivalent to that from 100 organisms was amplified and sequenced. We analyzed 10 *tprK* sequences from the low-passage harvest and 14 sequences from each of the medium- and high-passage harvests. Each of the 38 *tprK* sequences from Sea 81-4 is unique

across the seven V regions, pointing to the high level of sequence heterogeneity found in this gene; however, some individual V region sequences are shared among different *tprK* sequences. Among 38 Sea 81-4 clones, V1 and V4 are the least heterogeneous with three and nine different sequences, respectively (Fig. 3). V6 and V7 with 24 and 20 different sequences, respectively, have the highest level of heterogeneity in the Sea 81-4 isolate.

Figure 3 also illustrates that the accumulation of diversity differs among V regions. For example, two different V1 sequences are found at low passage, one new sequence is seen at medium passage, and no new sequences are found at high passage. In contrast, there are nine different V6 sequences at low passage, five new sequences are seen at medium passage, and ten additional sequences are found at high passage. Fur-

FIG. 2. Clustering analysis of *tprK* DNA sequences obtained directly from primary chancres of patients MD504, MD530, and MD55 (A) and from isolates that were propagated 4 (Sea 81-3), 6 (Sea 83-1), or 10 (Sea 84-2) times in rabbits (B). Cluster groups are made up of unique sequences from an individual patient or isolate, and are enclosed by a solid oval. Lowercase letters represent the unique sequences in each sample and, in panel A, numbers in parentheses indicate the number of sequenced plasmid clones that contain that sequence. In panel B, each plasmid clone contains a unique sequence. Trees were subject to 100 random bootstrap resamplings, and bootstrap numbers for major branches are shown. The marker bar represents a 1% difference in nucleotide sequences; within a given sample, there is greater genetic distance among rabbit-propagated sequences than among primary chancre sequences.

FIG. 3. Cumulative number of new V region sequences found in 38 *tprK* sequences from *T. pallidum* Sea 81-4 after low (▨), medium (▩), and high (■) passage.

ther analysis reveals that V regions with less diversity (V1, V2, V4, and V5) each have a common sequence found at all three passage levels. In contrast, no common sequence is found at all passage levels for the most diverse V regions (V3, V6, and V7). Accumulation of diversity is most clearly illustrated by detailed analysis of the founder *tprK* V4 sequence in Sea 81-4, TDVG HKKNGANGDIGAD (Table 2). This sequence is also seen, though in decreasing frequency, in the medium- and high-passage organisms. In the medium-passage organisms three new V4 sequences were identified, and five additional V4 sequences were identified in high-passage organisms. The amino acid changes predicted by these sequence changes are shown in Table 2.

DISCUSSION

Outer surface molecules of many bacterial pathogens undergo antigenic variation, allowing organisms to evade the host immune system and establish chronic infection. To produce a vast repertoire of serotypes, gene conversion occurs in *msp2* and *msp3* of the rickettsial pathogen *Anaplasma marginale* (2, 15) and in the *vsp* and *vlp* genes of *Borrelia hermsii*, the relapsing fever *Borrelia* spirochete (18, 22). The *vlsE* gene of the Lyme disease agent *Borrelia burgdorferi* also undergoes antigenic variation to produce a high level of diversity (23, 26, 27), and changes in the *VlsE* variable regions have been shown to alter *VlsE* antigenicity during infection (14). Similar to known mechanisms of antigenic variation in *Anaplasma* and *Borrelia*, our working model for antigenic variation of *tprK* is gene conversion from common donor sites.

Studies in our laboratories (16a, 17) have shown that antibodies to TprK arising from infection with the Nichols strain and other *T. pallidum* isolates are specifically targeted to the V regions, whereas T cells recognize epitopes in the conserved regions. Furthermore, immunization with recombinant Nichols TprK is partially protective against Nichols challenge in repeated studies (3, 16) but provides less protection against infectious challenge with *T. pallidum* strains expressing heterologous TprKs (16a). The major mode of clearance from early syphilis lesions is phagocytosis of opsonized treponemes (1, 9), and the subset of treponemes that remains at the site of infection after clearance is resistant to phagocytosis in vitro (12). Antiserum to TprK has been shown to be opsonic for *T. pal-*

lidum (3), suggesting that TprK is a target of immune clearance. We hypothesize that TprK heterogeneity may contribute to the ability of *T. pallidum* to evade the host immune response.

In the present study, we show that V1 is the least heterogeneous of the seven V regions in Sea 81-4. Previous studies in our laboratories demonstrated that, during infection with the Nichols strain and with non-Nichols isolates, antibodies are induced against all V regions except V1 (16a, 17). Taken together, these results suggest that V1 is under less immune pressure than the more heterogeneous V regions. V1 may also be less diverse than other V regions because it is involved in some necessary function for TprK. Nichols strain TprK transmembrane topology (3) and Kyte-Doolittle and TMpred tests of multiple TprK sequences from the Sea 81-4 isolate (data not shown) show that V1 is located in a hydrophilic region of the protein; nonetheless, V1 may be topologically hidden or otherwise inaccessible to B cells, thus restricting exposure of V1 to the immune system.

Further evidence that V regions have different levels of diversification is provided by our analysis of *tprK* sequences from the *T. pallidum* isolate Sea 81-4 after low, medium, and high passages. Sequences from V regions with low to moderate heterogeneity, such as V4, were maintained at all passage levels, whereas no identical sequences from V regions with high heterogeneity were found at all three passage levels. Because treponemes were harvested at the peak of infection and before the initiation of significant immune clearance, this analysis suggests that V regions have different intrinsic rates of diversification.

We found that *tprK* sequences in primary chancres are less diverse than those in rabbit-propagated isolates. Early studies on the transmission of *T. pallidum* showed that intradermal injection of as few as 10 organisms may lead to infection in humans (13). Syphilis transmission occurs primarily during early infection when numbers of treponemes are high; however, the replication rate of *T. pallidum* is quite slow, and the average time to produce a primary chancre in the exposed individual is 2 to 6 weeks, suggesting that the infectious dose is low. We propose that a very low number of treponemes is transmitted in natural infection and that treponemes in early

TABLE 2. Predicted TprK V4 amino acid sequences from *T. pallidum* Sea 81-4

Predicted amino acid sequence ^a	No. (%) of sequences		
	Low passage (n = 10)	Medium passage (n = 14)	High passage (n = 14)
TDVGHKKNGANGDIGAD	10 (100)	8 (57)	6 (43)
TDVGHKKNGA <u>Q</u> GT <u>V</u> GAD		4 (29)	1 (7)
TDVGHK <u>R</u> NGANGDIGAD		1 (7)	0 (0)
TDVGHKKNGA <u>D</u> GDIGAD		1 (7)	0 (0)
TDVGR <u>K</u> K <u>D</u> GAQGT <u>V</u> GAD			2 (14)
TDVGR <u>K</u> K <u>D</u> GANGDIGAD			1 (7)
TDVGHKK <u>E</u> NAANGDIGAD			1 (7)
TDVGHKK <u>E</u> NA <u>A</u> PDDIGAD			1 (7)
TDVGHKK <u>E</u> NA <u>A</u> NVNGT <u>V</u> GAD			2 (14)

^a Amino acid changes in medium-passage sequences are single underlined; amino acid changes in high-passage sequences are double underlined.

chancres (at the time of transmission) have not yet been subjected to significant immune selection. This is reflected in the clustering analysis showing that *tpk* sequences are more related within infected individuals than among individuals. This result suggests that treponemes in individuals are derived from a limited number of transmitted organisms with related *tpk* sequences.

The Sea 81-3, Sea 83-1, and Sea 84-2 isolates were generated by infecting rabbits with cerebrospinal fluid from syphilis-infected patients and are likely to have arisen from very low numbers of treponemes, just as in the directly sequenced chancre samples. After rabbit passage, however, treponemes within each rabbit-propagated isolate are more diverse than those within treponemes from primary chancres, as indicated by the greater genetic distance between sequences. In addition, sequences from Sea 81-3, which was passaged 4 times in rabbits, cluster more tightly than sequences from Sea 84-2, which was passaged 10 times. In contrast to transmission of low numbers of organisms in nature, *T. pallidum* is propagated in the laboratory by passaging 10^6 to 10^8 organisms to naive rabbits. Harvesting occurs at the peak of infection and before significant immune clearance begins; therefore, as in natural infection, there is not yet any obvious effect of immune selection. Because the number of organisms passaged in laboratory propagation of *T. pallidum* is likely to be much higher than in human transmission, rabbit propagation may afford a greater opportunity for accumulation of diversity in *tpk* than does natural infection.

Our laboratories have generated strains of *T. pallidum* that carry a single *tpk* sequence, and we are placing these strains under immune pressure by allowing immune clearance in vivo before passaging. In addition to investigating generation of *tpk* upon passage of clonal strains, we will also directly determine whether *tpk* varies in a single infection by examining sequences during early and late infection. These experiments will test whether certain V regions undergo stronger immune selection than others and will provide further evidence for *tpk* diversification as a mechanism of antigenic variation. The expression of new TprK proteins may contribute to the ability of *T. pallidum* to persist in the face of a robust immune response and to reinfect previously exposed individuals.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI34616 and AI42143 (to S.A.L.), AI43456 (to W.C.V.V.), and AI45724 (to A.M.R.). R.E.L. was supported by NIH training grant AI07140.

We thank Timothy Rose and John Mittler for helpful discussions and Heidi Pecoraro for manuscript preparation.

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