J. Protozool. Res., 8, 204-213 (1998)

Diagnosis of *Trypanosoma evansi* by the polymerase chain reaction (PCR)

J. E. DONELSON[1] and W.T. ARTAMA[2]

[1]*Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242, USA, and* [2]*IUC Biotechnology, University of Gadjah Mada, Fl. Teknika Utara, Barek, Yogyakarta, INDONESIA*

Received 20 August 1998

Key words: *Trypanosoma evansi*; diagnosis; polymerase chain reaction

ABSTRACT

Trypanosoma evansi and the three subspecies of *T. brucei* are indistinguishable from each other morphologically and are thought to be very closely related evolutionarily. The most notable molecular difference between *T. evansi* and the *T. brucei* subspecies is the structure of the DNA molecules found in their kinetoplast, a subcellular organelle located near the flagellar pocket. We have exploited this difference in kDNA sequence and organization to develop a polymerase chain reaction (PCR)-based assay that can distinguish between *T. evansi* and *T. brucei*. Oligonucleotide primers spaced about 370 bp apart on the *T. evansi* kDNA sequence generate a PCR-amplification product when total *T. evansi* DNA is used as the template, whereas no amplification product occurs when total DNA from *T. brucei* or *Leishmania* is the template. In contrast, when PCR primers specific for three different nuclear DNA sequences of *T. brucei* - a 177-bp repeat, the procyclin gene or the spliced leader gene - are used in similar PCR amplifications, identical PCR products are obtained from the *T. brucei* and *T. evansi* template DNAs. Thus, the nuclear DNA sequences of *T. evansi* and *T. brucei* are very similar, but their kDNAs are sufficiently different to serve as the basis for PCR-based diagnosis of the two trypanosome species.

INTRODUCTION

Trypanosoma evansi is the species of trypanosomes that causes 'surra', a wasting disease of domestic livestock. This protozoan parasite is transmitted mechanically from one animal to another by biting flies such as the tabanids, but it does not appear to possess any specific developmental stages in these insect vectors. Morphologically, *T. evansi* is indistinguishable from the three *T. brucei* subspecies (spp.) which undergo several developmental stages in African tsetse flies and cause the fatal diseases of sleeping sickness in humans or nagana in domestic livestock. The general similarity between *T. evansi* and *T. brucei* was noted 100 years ago by Kanthack, Durham and Blandford (1898) who commented that "The Haematozoon of nagana has already been described by Bruce, and is closely allied to the *Trypanosoma* of surra" (Stephen 1986). More recently, isoenzyme analysis and nuclear DNA polymorphisms (Gibson, Wilson and Moloo 1983; Masiga and Gibson 1990) have been used to demonstrate this close relationship between the two trypanosome species on a molecular level.

In addition to the developmental differences between *T. evansi* and the *T. brucei* spp. in their transmitting insects, they are also known to differ in the molecular structure of the DNA molecules within their cytoplasmically located kinetoplast. In the *T. brucei* spp. this kinetoplast DNA (kDNA) is a large interlocked network of about 10,000 copies of 1-kb minicircle DNAs of heterogeneous sequences, and about 50 copies of 23-kb maxicircle DNAs which have a common sequence that is genetically equivalent to mitochondrial DNA of other organisms (Borst et al. 1976; Simpson 1986). In contrast, the kDNA networks of

T. evansi lack maxicircle DNAs completely and possess only interlocked 1-kb minicircle DNAs that appear to have the same, or nearly the same, sequence (Borst, Fase-Fowler and Gibson 1987).

We set out to determine if specific nucleotide sequences in the nuclear DNA or minicircle kDNA of *T. evansi* and the *T. brucei* spp. could be identified that would permit the use of the polymerase chain reaction (PCR) to distinguish between these two species. In the case of nuclear DNA, we expected that *T. brucei* repetitive DNA sequences or *T. brucei* nuclear genes known to be expressed only during a specific developmental stage in tsetse flies might be missing in the *T. evansi* genome. On the kDNA level, we anticipated that the single *T. evansi* minicircle sequence, or portions of that sequence, might not be represented among the many heterogeneous sequences in the *T. brucei* minicircles. We found, somewhat surprisingly, that the nuclear repetitive sequences and tsetse fly stage-specific genes of *T. brucei* also occur in the *T. evansi* genome, rendering them unsuitable for PCR diagnosis, but did find that the *T. evansi* minicircle DNA sequence can be used in PCR amplifications to distinguish between *T. evansi* and *T. brucei*. Most of the data described here have been presented in an earlier publication (Artama, Agey and Donelson 1992).

METHODS and MATERIALS

Polymerase chain reactions (PCRs): PCR amplifications were conduced in a 50 μl reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 8% dimethysulphoxide, 200 μM each of the four dNTPs, 10 (M each of the oligonucleotide primers, 10 ng of total genomic DNA and 1.25 units of *Taq* (*Thermus aquaticus*) DNA polymerase (Perkin-Elmer Cetus, Norwalk, Connecticut, USA). The amplifications were conducted for 20 cycles in a DNA Thermal Cycler (Perkin-Elmer Cetus) according to the following three-step regiment: 94℃ for 1 min (denature), 55℃ for 2 min (anneal) and 72℃ for 2 min (extension). For the spliced leader (SL) gene amplifications the annealing temperature was 50℃ instead of 55℃.

The oligonucleotide primer sets used for the PCR amplifications were:

SL gene primers:
5'-TAGCGTTAGTTGAAAGC-3' and
5'-TATTATTAGAACAGTTTCTGTAC-3';
177-bp repeat primers:
5'-CGAATGAATATTAAACAATGCGCAGT-3' and
5'-AGAACCATTTATTAGCTTTGTTGC-3';
procyclin gene primers:
5'-CACAATGGCACCTCGTTCCC-3' and
5'-TTAGAATGCGGCAACGAGA-3';
minicircle primers:
5'-CAACGACAAAGAGTCAGT-3' and
5-ACGTGTTTTGTGTATGGT-3'.

Genomic DNA isolations: Primary isolates of *T. evansi* were obtained from the blood of two infected water buffalo in Kulon Progo district, Yogyakarta, Indonesia, as described previously (Artama, Agey and Donelson 1992). A bloodstream *T. evansi* clone was isolated from each of the two primary isolates by passage through laboratory rabbits followed by

serial dilution to about 1 parasite/400 μ l solution and passage through X-irradiated laboratory mice, also as previously described (Artama, Agey and Donelson 1992). The two bloodstream clones were named YogTat 1.1 and YogTat 2.1.

The two *T. evansi* clones were grown in X-irradiated mice, purified by DEAE column chromatography (Lanham and Godfrey 1970) and their total genomic DNA isolated as described (Laurent, Van Assel and Steinert 1971). Genomic DNAs from the African trypanosome *T. b. rhodesiense* (Lenardo et al. 1984), the Latin American trypanosome *T. cruzi* (Silva and Nussenzweig 1953) and *Leishmania donovani*, Sudan S1 strain (F. Neva, unpublished observations) were generously provided by Ying Lu, Kwang S. Kim and Louis V. Kirchhoff at the University of Iowa. Salmon sperm DNA was purchased from Bethesda Research Labs, Bethesda, Maryland.

Minicircle DNA cloning and sequencing: Total DNA (20 μ g) from *T. evansi* clone YogTat 1.1 was digested to completion with *Sau*3A and the fragments separated on a 0.8% agarose gel (FMC Bioproducts, Rockland, Maine) followed by ethidium bromide staining. A smear of DNA fragments was detected from which loomed a band of DNA fragments whose sizes corresponded to a standard length DNA marker of 1 kb. The DNA fragments in this band were electroeluted, ligated into the unique *Bam*HI site of plasmid pBluescript and the recombinant plasmids transformed into *Escherichia coli* (Sambrook, Fritsch and Maniatis 1989). The recombinant plasmids in three *E. coli* transformants were purified and found by partial DNA sequence determination to contain the same DNA insert. The complete sequence of one of the inserts was determined by the step-wise procedure of end-sequencing followed by the use of new primers whose sequences were located about 250 nucleotides downstream of the previous primers.

RESULTS

PCR amplifications of the repetitive spliced leader (SL) genes and 177-bp repeats of T. brucei: One of the many unique features of Trypanosomatids is the presence of a 39-nucleotide SL sequence at the 5' ends of all of their mRNAs (Walder et al. 1986). This SL, whose sequence differs slightly among the different Trypanosomatids, is encoded by

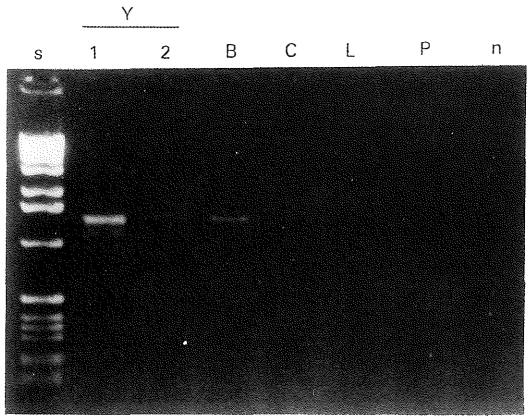

Figure 1. Agarose gel stained with ethidium bromide showing the PCR amplification products of the SL primers using as the template total DNA from *Trypanosoma evansi* clones YogTat 1.1 (Y1) and YogTat 2.1 (Y2), a *T. brucei* clone (B), a *T. cruzi* clone (C), *Leishmania donovani* (L), salmon sperm (P) or no DNA (n). Lane s contains standard length DNA molecules.

tandemly repeated genes that are transcribed into precursor RNAs which donate the SL in a trans-RNA splicing reaction to the 5' ends of all RNAs destined to become mature mRNAs (Nilsen 1995). In the *T. brucei* genome there are about 200 SL genes located within 1.35 kb repeats. The SL genes of *T. evansi* have not been examined so we used PCR amplifications to investigate whether these genes were similarly organized in *T. evansi* and

the *T. brucei* spp. Figure 1, lane B, shows that when *T. brucei* genomic DNA serves as a template for a PCR amplification with oligonucleotide primers specific for the SL gene repeat, an amplification product of 1.35 kb is obtained as expected. Likewise, when genomic DNA from either *T. evansi* clone YogTat 1.1 or YogTat 2.1 is used as the template, the same sized amplification product occurs (lanes Y1 and Y2 respectively). A weak amplification product of a different size can also be observed in the *T. cruzi* DNA lane, but none occurs in the *L. donovani* or salmon sperm DNA lanes, indicating that this primer set does not hybridize sufficiently to these DNAs to generate a detectable amplification product. Collectively, these results demonstrate that the genomic DNA repeat containing the SL gene in *T. evansi* is similar, if not the same, as that in *T. brucei.*

Another tandemly repeated sequence in the nuclear DNA of the *T. brucei* spp. is a sequence of 177 bp that occurs in the one hundred or more minichromosomes in the nucleus - small linear DNA molecules of 50 to 150 kb that serve as repositories of telomere-linked genes for the variant surface glycoproteins (VSGs). No other genes have been detected in the minichromosomes and no other function have been ascribed to them.

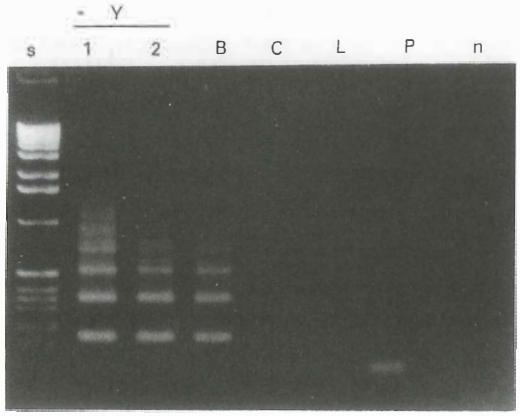

Figure 2. Agarose gel stained with ethidium bromide showing the PCR amplification products of primers specific for the 177-bp nuclear repetitive DNA of *T. brucei.* The template DNAs were the same as those indicated in Fig. 1.

The center portions of these minichromosomes are thought to be composed primarily of 177-bp repeats, of which more than 10,000 copies exist in the haploid genome (Sloof et al. 1983). Figure 2 shows a ladder of 177-bp PCR products that are obtained when primers that hybridize to the 177 bp are used for amplification of template DNA from either *T. brucei* or *T. evansi.* The ladder results from the primers being extended through multiple tandem copies of this short repeat during each PCR cycle. If the extension time of the reaction is reduced, the higher multimers of the 177 bp disappear (not shown). Elution of the 177-bp repeat band from the YogTat 1.1 lane and direct sequence determination of the mixture of fragments from both ends revealed the same basic sequence as reported for *T. brucei.* Several nucleotide positions on the sequencing gels contained multiple bands on both strands, indicating the presence of nucleotide heterogeneity at these positions, a finding consistent with the earlier studies of the *T. brucei* sequence (Sloof et al. 1983). Figure 2 also shows that DNA from *T. cruzi* and *L. donovani* (lanes C and L) do not serve as templates for this primer set. Salmon sperm DNA (lane P) does serve as a template for a very small PCR product that was not investigated further.

PCR amplification of the procyclin genes of the T. brucei spp: Procyclin is a well-studied abundant protein that occurs on the surface of *T. brucei* during the procyclic stage development in the tsetse fly (Roditi et al. 1998). Although its precise function is not known, it is thought to participate in the attachment of procyclic trypanosomes to the lumen

of the insect gut. There are about 10 million copies of procyclin on the procyclic trypanosome surface and it is the only protein known to be present on the surface during this developmental stage. Since *T. evansi* is transmitted mechanically and does not appear to undergo different developmental stages in its insect vectors, we decided to search for the existence of procyclin genes in its genome using PCR primers derived from the procyclin coding sequence. Figure 3 shows that the presence and sizes of the *T. brucei* procyclin genes are completely preserved in *T. evansi*. The multiple bands in the 300-400 bp range represent different procyclin genes. The largest amplification band in the YogTat 1.1 and *T. brucei* lanes is likely generated by amplification across two adjacent procyclin genes. A similar faint band, not apparent in the photograph, was present in the YogTat 2.1 lane on the actual stained gel. When the same procyclin-specific primers were used on DNA from *T. cruzi*, *L. donovani* and salmon sperm DNA, no PCR amplification product was generated (not shown). Thus, genes for procyclin occur in the *T. evansi* genome despite the fact that procyclin is not known to be required during the *T. evansi* life cycle.

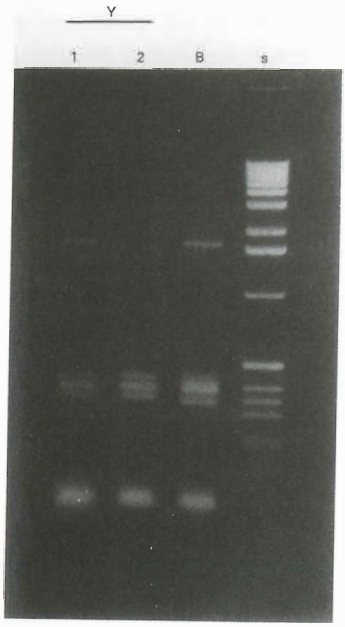

Figure 3. Agarose gel stained with ethidium bromide showing the PCR amplification products of primers specific for the procyclin genes of *T. brucei* and template DNAs from *T. evansi* clones YogTat 1.1 (Y1) and 2.1 (Y2) and *T. brucei* (B). Lane s contains standard length DNA molecules.

Determination of the T. evansi kDNA minicircle sequence: At the time this work was begun, it was thought that, in contrast to the *T. brucei* spp., the kDNA minicircles of *T. evansi* all contained the same, or nearly the same, nucleotide sequence (Borst, Fase-Fowler and Gibson 1987). Since this sequence had not yet been determined, we cloned and sequenced several kDNA minicircle molecules from *T. evansi* clone YogTat 1.1. In anticipation that the 1-kb band observed in an agarose gel of a *Sau*3A digest of total DNA from clone YogTat 1.1 (see Methods and Materials) contained the linearized kDNA minicircles, the fragments in this band were eluted and cloned into plasmid pBluescript. The complete 994-bp sequence of one cloned insert was determined and is shown in Figure 4A. Portions of two other cloned 1-kb inserts were sequenced and were identical in the sequenced regions to the one that was completely sequenced.

Several features of the sequence are similar or identical to minicircle sequences reported in other Trypanosomatids (Simpson 1986) and in particular to those described for *T. brucei* spp. (Chen and Donelson 1980; Jasmer and Stuart 1986a). The 13 bp sequence thought to be the minicircle replication origin (Ntambi and Englund 1985) occurs within a 120 bp

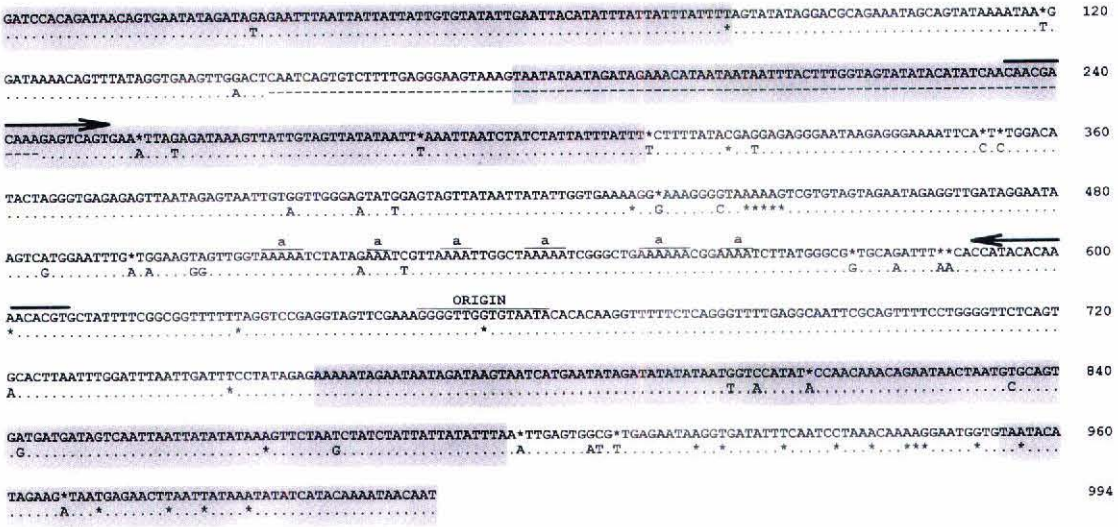

Figure 4. (A) Comparison of the nucleotide sequence of a cloned minicircle DNA from *T. evansi* YogTat 1.1 (top strand, GenBank accession no. M81584) and the *T. evansi* RoTat 2/1 minicircle DNA sequence (bottom strand, accession no. M34848) (Songa et al. 1990). The YogTat 1.1 sequence begins at a unique *Sau*3A site. Dots indicate positions of nucleotide identity, asterisks show deletions introduced to maximize alignment, and dashes represent a potential *Hinf*I fragment of the RoTat 2/1 minicircle whose sequence was not reported (Songa et al. 1990). Horizontal arrows show the sequences of sense and anti-sense oligonucleotides used as primers for the PCR amplifications shown in Fig.5. Shaded regions enclose three regions that are flanked by variations of the 18-bp inverted repeats found in *T. brucei* minicircle DNAs (Jasmer and Stuart 1986b; Pollard et al. 1990). The letter 'a' above the horizontal lines indicates several short runs of adenosine residues. ORIGIN shows the conserved sequence shown to be the replication origin in kDNA minicircles of other Trypanosomatids.

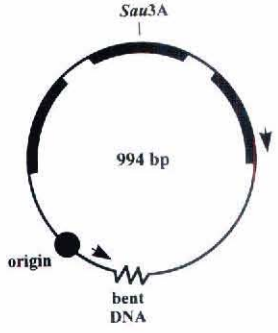

Figure 4. (B) Schematic diagram of the YogTat 1.1 minicircle sequence showing the F positions of the unique *Sau*3A site, the potential replication origin (origin), the three cassettes flanked by the 18 bp repeat variations (thick lines), a potential bent DNA region (bent DNA), and binding sites for the PCR primers (arrows).

region that has substantial homology (not indicated) with the conserved region of *T. brucei* minicircles (Chen and Donelson 1980). Several short runs of adenosine residues are present in one region that likely corresponds to the "bent DNA" portion of other minicircles (Marini et al. 1982). Three separate segments of about 140 nucleotides each are flanked by variations of 18 bp inverted repeats (Jasmer and Stuart 1986b). Thus, the sequence is very likely to be derived from the kinetoplast of YogTat 1.1 trypanosomes. The locations of these common minicircle features in the YogTat 1.1 sequence are summarized in Figure. 4B. While the sequence determination was underway, the partial sequence of a minicircle from another Indonesian *T. evansi* clone, RoTat 2/1, was published (Songa et al. 1990) and this sequence is compared with the YogTat 1.1 sequence in Figure 4B. Of the approximately

890 nucleotides in which the two sequences can be directly compared, they have 93% identity. The most distinctive difference among the two sequences is the presence of scattered deletions in the RoTat 2/1 sequence relative to the YogTat 1.1 minicircle, including one in the putative 13-mer replication origin. Both of these sequences are very similar to the partial or complete minicircle sequences of *T. evansi* from several other areas of the world (Songa et al. 1990; Ou and Baltz 1991). Thus, it seems clear that a relatively unique minicircle sequence is conserved in most *T. evansi* isolates around the world, in contrast to the highly heterogeneous population of *T. brucei* minicircles, both within single *T. brucei* clones and among different isolates (Donelson, Majiwa and Williams 1979).

PCR amplification of the T. evansi minicircle: Two of the oligonucleotides synthesized for the dideoxy sequence determinations were used as primers in PCR amplifications of total DNA from *T. evansi* and several other organisms. One primer hybridizes within the 120 bp "origin" region that has considerable sequence similarity between *T. evansi* and *T. brucei* minicircles (see above), and the other primer binds within one of the three segments flanked by the variations of the 18 bp repeats (Fig. 4). These PCR primers generate the expected minicircle amplification product from *T. evansi* total DNA, but somewhat surprisingly, under the conditions used do not amplify the minicircle sequence from *T. brucei* or the other organisms (Fig. 5). Control restriction digests of the *T. brucei* DNA and trial amplifications of DNAs from other *T. brucei* spp. isolates available to us demonstrated that the minicircle DNAs were present in the total DNA preparations but did not serve as templates for the PCRs with these primers. Furthermore, inspection of the partial or complete minicircle DNA sequences from other *T. evansi* isolates (Songa et al. 1990; Ou and Baltz 1991) suggest that this primer set will amplify the minicircle sequence from the other isolates as well. Thus, these two PCR primers appear to be specific for *T. evansi*, but not for *T. brucei*. Since one of the primers is derived from a region conserved between *T. evansi* and *T. brucei* minicircles, the simplest interpretation of the results is that a complementary sequence for the other primer does not exist within the heterogeneous population of minicircles in the *T. brucei* spp.

Figure 5. Agarose gel stained with ethidium bromide showing the PCR amplification products of the YogTat 1.1 minicircle PCR primers (see Fig. 4) using as templates the total DNAs from *T. evansi* clones YogTat 1.1 (Y1) and YogTat 2.1 (Y2)., *T. brucei* (B), *L. donovani* (L), salmon sperm (P) or no DNA (n). Lane s contains a 1-kb ladder of standard length DNA molecules. The 1-kb marker is the isolated band near the center of the photograph in lane s.

DISCUSSION

The DNA characterizations described here support the original predictions of a close evolutionary relationship between these two species of salivarian trypanozoan (Stephen 1986). In particular, the consensus sequence of the 177 bp nuclear DNA repeat is identical in the two organisms, and the PCR amplification pattern of procyclin genes is the same. Likewise, the spliced leader gene repeat appears to be very similar in the two organisms.

Of the DNA sequence features examined, the most differences appear in the minicircle sequences. These differences are of interest for two reasons. First, the PCR amplifications shown in Figure 5 suggest that minicircle-specific primers can be used to distinguish between *T. evansi* and *T. brucei* spp. in PCR-based assays. The examination of a wider range of *T. brucei* spp. isolates than is available to us will be necessary to confirm this PCR result, but if it can indeed be extended, similar PCR amplifications could serve as an important diagnostic tool in portions of Africa where overlap between *T. evansi* and *T. brucei* spp. exists (Gibson, Wilson and Moloo 1983; Masiga and Gibson 1990). In Indonesia and other regions of Southeast Asia, similar PCR-based assays using any of the primer sets described here have the potential of providing a much more thorough survey of *T. evansi* distribution and prevalence than currently is available.

The minicircle sequence comparisons are also of interest because the heterogeneous *T. brucei* minicircles are transcribed into small RNA molecules which can potentially serve as templates for guiding insertions and deletions of uridines in maxicircle transcripts, a post-transcriptional process called RNA editing (Benne 1989; Pollard et. al. 1990; Alfonzo, Thiemann and Simpson 1997). The only known function of minicircles is to encode such guide RNAs (gRNAs), a role that seems unnecessary in *T. evansi* which does not possess maxicircle DNA (Borst, Fase-Fowler and Gibson 1987). Another salivarian trypanozoan with a homogeneous minicircle sequence is *T. equiperdum*, which is transmitted venereally among horses in the absence of an insect vector (Barrois, Riou and Galibert 1981). However, in contrast to *T. evansi*, this organism appears to possess maxicircle DNA, albeit with a deletion that may have eliminated several protein-encoding genes (Frasch et al. 1980). The *T. equiperdum* minicircle undergoes transcription from three regions within 18 bp inverted repeats, similar to the *T. brucei* minicircle transcripts, suggesting that these transcripts also may serve as gRNAs (Pollard and Hajduk 1991).

When the *T. evansi* and *T. equiperdum* minicircle sequences are compared (not shown), 94% identity occurs within a 140 bp region containing the putative replication origin and the adjacent "bent DNA", whereas the remainder of the sequences share about 50% identity. The 50% identity extends through the three segments flanked by the variations of the 18 bp repeats. When the sequences between these 18 bp inverted repeats in *T. evansi* are compared by computer analysis with the *T. brucei* maxicircle sequences, the largest region of complete complementarily allowing G/T base pairs is 10 nucleotides, considerably fewer than the 30-50 nucleotides found in *T. brucei* minicircles (Pollard et al. 1990). Although this negative finding does not contribute to an understanding of why the *T. evansi* minicircle sequence has been maintained, it may suggest that providing gRNAs is not the reason.

Finally, it should be noted that the 23-kb kDNA maxicircle sequence of *T. brucei* could also serve as a template in PCR amplification to distinguish between *T. evansi* and the *T. brucei* spp. In this case, a PCR product should occur when *T. brucei* DNA is the template, but no product should appear when *T. evansi* DNA is the template because of the lack of kDNA maxicircles in *T. evansi*. This result would be the opposite of that shown in Figure 5 where a PCR product is obtained with *T. evansi* template DNA, but not with *T. brucei*

template DNA.

ACKNOWLEDGEMENTS

During this work W.T.A. was the recipient of a travel award from the Indonesian Second University Development Project. The University of Iowa DNA Core Facility is supported by National Institutes of Health grant DK25295.

REFERENCES

Alfonzo, J.D., Thiemann, O. & Simpson, L. 1997. The mechanism of U insertion/deletion RNA editing in kinetoplastid mitochondria. *Nucl. Acids. Res. 25: 3751-3759.*

Artama, W.T., Agey, M.W. & Donelson, J. E. 1992. DNA comparisons of *Trypanosoma evansi* (Indonesia) and *Trypanosoma brucei* spp. *Parasitology 104: 67-74.*

Barrois, M., Riou, G. & Galibert, F. 1981. Complete nucleotide sequence of minicircle kinetoplast DNA from *Trypanosoma equiperdum. Proc. Natl Acad. Sci., USA 78: 3323-3327.*

Benne, R. 1989. RNA editing in trypanosome mitochondria. *Biochim. Biophys. Acta. 1007: 131-139.*

Borst, P., Fase-Fowler, F. & Gibson, W.C. 1987. Kinetoplast DNA of *Trypanosoma evansi. Mol. Biochem. Parasitol. 23: 31-38.*

Bort, P., Fairlamb, A.H., Fase-Fowler, F, Hoeijmakers, J.H. & Weislogel, P.O. 1976. The structure of kinetoplast DNA. pp. 59-69. In: *The Genetic Function of Mitochondrial DNA, (ed. Saccone, C. & Kroon, A.M.). Amsterdam, North-Holland.*

Chen, K.K. & Donelson, J.E. 1980. Sequences of two kinetoplast DNA minicircles of *Trypanosoma brucei. Proc. Natl Acad. Sci., USA 77: 2445-2449.*

Donelson, J.E. 1995. Mechanisms of antigenic variation in *Borrelia hermsii* and African trypanosomes. *J. Biol. Chem. 270: 7783-7786.*

Dorfman, D.M. & Donelson, J.E. 1984. Characterization of the 1.35 kb DNA repeat unit containing the conserved 35 nucleotides at the 5' termini of variant surface glycoprotein mRNAs in *Trypanosoma brucei. Nucl. Acids Res. 12: 4907-4920.*

Donelson, J.E., Majiwa, P.A.O. & Williams, R.O. 1979. Kinetoplast DNA minicircles of *Trypanosoma brucei* share regions of sequence homology. *Plasmid 2: 572-588.*

Frasch, A.C.C., Hajduk, S.L., Hoeijmakers, J.H.H., Borst, P., Brunel, F. & Davison, J. 1980. The kinetoplast DNA of *Trypanosoma equiperdum. Biochim. Biophys. Acta 607: 397-410.*

Gibson, W.C., Wilson, A.J. & Moloo, S.K. 1983. Characterization of *Trypanosoma (Trypanozoon) evansi* from camels in Kenya using isoenzyme electrophoresis. *Res. Vet. Sci. 34: 114-118.*

Hattori, M. & Sakaki, Y. 1986. Dideoxy sequencing method using denatured plasmid templates. *Anal. Biochem. 152: 232-238.*

Jasmer, D.P. & Stuart, K. 1986a. Conservation of kinetoplastid minicircle characteristics without nucleotide sequence conservation. *Mol. Biochem. Parasitol. 18: 257-269.*

Jasmer, D.P. & Stuart, K. 1986b. Sequence organization in African trypanosome minicircles is defined by 18 bp inverted repeats. *Mol. Biochem. Parasitol. 18: 321-331.*

Kanthack, A.A., Durham, H.E. & Blandford, W.F.H. 1898. On nagana or tsetse-fly disease. *Proc. Royal Soc. 64: 100.*

Kirchhoff, L.V., Kim, K.S., Engman, D.M. & Donelson, J.E. 1988. Ubiquitin genes in Trypanosomatidae. *J. Biol. Chem. 263: 12698-12704.*

Lanham, S.M. & Godfry, D.G. 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Exptl Parasitol. 28: 521-534.*

Laurent, M., Van Assel, S. & Steinert, M. 1971. A unique macromolecular structure of considerable size and mechanical resistance. *Biochem. Biophys. Res. Com. 43: 278-284.*

Lenardo, M.J., Rice-Ficht, A.C., Kelly, G., Esser, K. & Donelson, J.E. 1984. Characterization of the genes specifying two metacyclic variant surface antigen types in *Trypanosoma brucei* rhodesiense. *Proc. Natl Acad. Sci., USA 81: 6642-6646.*

Marini, J.C., Levene, S.D., Crothers, D.M., & Englund, P.T. 1982. Bent helical structure in kinetoplast DNA. *Proc. Natl Acad. Sci., USA 79: 7664-7668.*

Masiga, D.,K. & Gibson, W.C. 1990. Specific probes for *Trypanosoma (Trypanozoon) evansi* based on kinetoplast DNA minicircles. *Mol. Biochem. Parasitol. 40: 279-284.*

Moser, D.R., Cook, G.A., Ochs, D.E., Bailey, C.P., McKane, M.R. & Donelson, J.E. (1989). Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using the polymerase chain reaction. *Parasitology 99: 57-66.*

Nilsen, T.W. 1995. Trans-splicing: an update. *Mol. Biochem. Parasitol. 73: 1-6.*

Ntambi, J.M. & Englund, P.T. 1985. A gap at the unique location in newly replicated kinetoplast DNA minicircles from *Trypanosoma equiperdum. J. Biol. Chem. 260: 5574-5579.*

Ou, Y.C . & Baltz, T. 1991. Kinetoplast DNA analysis of four *Trypanosoma evansi* strains. *Mol. Biochem. Parasitol. 46: 97-102.*

Pollard, V.W. & Hajduk, S.L. 1991. *Trypanosoma equiperdum* minicircles encode three distinct primary transcripts which exhibit guide RNA characteristics. *Mol. Cell. Biol. 11: 1668-1675.*

Pollard, V.W., Rohrer, S.P., Michelotti, E.F., Hancock, K. & Hajduk, S.L. 1990. Organization of minicircle genes for guide RNAs in *Trypanosoma brucei. Cell 63: 783-790.*

Roditi, I., Furger, A., Ruepp, S., Schürch, N. & Bütikofer, P. 1998. Unravelling the procyclin coat of *Trypanosoma brucei. Mol. Biochem. Parasitol. 91: 117-130.*

Sambrook, J., Fitsch, E.F. & Maniatis, T. 1989. Molecular Cloning: *A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.*

Silva, L.H.P. & Nussenzweig, V. 1953. Sobre uma cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. (On a strain of *Trypanosoma cruzi* highly virulent for white mice). *Folia Clinical Biologie 20: 197-207.*

Simpson, L. 1986. Kinetoplast DNA in trypanosomatid flagellates. *International Rev. Cytol. 99, 119-179.*

Sloof, P., Menke, H.H., Caspers, M.P. & Borst, P. 1983. Size fractionation of *Trypanosoma brucei* DNA: localization of the 177-bp repeat satellite DNA and a variant surface glycoprotein gene in a minichromosomal DNA fraction. *Nucl. Acids Res. 11: 3889-3901.*

Songa, E. B., Paindavoine, P., Wittouck, E., Viseshakul, N., Muldermans, S., Steinert, M. & Hamers, R. 1990. Evidence for kinetoplast and nuclear DNA homogeneity in *Trypanosoma evansi* isolates. *Mol. Biochem. Parasitol. 43: 167-180*

Stephen, L. E. 1986. In: Trypanosomiasis, a veterinary perspective. *Pergamon Press, Oxford, pp. 184-215.*

Walder, J.A., Eder, P.S., Engman, D.M., Brentano, S.T., Walder, R.Y., Knutzon, D.S., Dorfman, D.M. & Donelson, J.E. 1986. The 35 nucleotide spliced leader sequence is shared by all trypanosome messenger RNAs. *Science 233: 569-571.*