J. Protozool. Res., 8, 28-43 (1998)

Kinetoplast DNA and Procyclic Acidic Repetitive Protein A-α Gene of *Trypanosoma evansi*

N. INOUE[1], Y. HONZAKO[2], K. HIRUMI[1], X. XUAN[1], T. AGATSUMA[2], H. NAGASAWA[1], T. MIKAMI[1], AND H. HIRUMI[1]

[1] *The Research Center for Protozoan Molecular Immunology,* [2] *Department of Bioresource Chemistry, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan.*

Received 10 June 1998 / Accepted 16 August 1998

Key words : PARP, kDNA, *Trypanosoma evansi*, *T. brucei*

ABSTRACT

Trypanosoma evansi is morphologically indistinguishable from *T. brucei*. Close relationship between *T. evansi* and *T. brucei* was further documented by Gibson, Wilson and Moloo (1983), Masiga and Gibson (1990) and Stevens et al. (1992), suggesting that *T. evansi* is derived from *T. brucei*. However, *T. evansi* has been distinguished from *T. brucei* by their lack of maxicircle DNA, minicircle DNA homogeneity and lack of developmental stages in its insect vectors (Stuart 1983). Although several features of the minicircle DNA sequence have been reported to be specific for *T. evansi* (Masiga et al. 1990; Artama, Agey and Donelson 1992), we report here that two strains of *T. brucei* (*T. b. gambiense* Welcome and *T. b. rhodesiense* IL1501) also possess "the *T. evansi* specific" minicircle DNA sequence.

Furthermore, Artama et al. (1992) have reported a similarity of PCR amplification patterns of the procyclic acidic repetitive protein (PARP), which is known to be a major surface protein of procyclic forms of *T. brucei* (Mowatt and Clayton 1987; Richardson et al. 1988), between *T. evansi* and *T. brucei*. In this study, we confirmed the existence of the similarity and suggested the use of the PARP primer as a diagnostic tool for the *T. evansi* infection. Although the PCR detection using this primer will not still distinguish the *T. evansi* infection from the *T. brucei* infection, it will be specially useful outside of the tsetse belt in where both the kinetoplastic and akinetoplastic strains of *T. evansi* are widely prevalent

but not *T. brucei*.

Moreover, the PARP A-α gene of *T. evansi* was sequenced, and the expression of the PARP gene in *T. evansi* was, for the first time, demonstrated by means of RT-PCR, although *T. evansi* is lacking the procyclic stage in its life cycle.

INTRODUCTION

Trypanosoma evansi is a causative agent of surra which is a wasting disease of domestic animals in South America, Northern Africa, Middle East and Asia. The parasite is transmitted mechanically by biting flies, such as Tabanidae and *Stomoxys*, and vampire, and does not have any developmental stages in its insect vectors, and its bloodstream forms are morphologically indistinguishable from those of *T. brucei*. Close relationship between *T. evansi* and *T. brucei* was further documented by Gibson et al. (1983), Masiga et al. (1990) and Stevens et al. (1992), suggesting that *T. evansi* has been derived from *T. brucei*.

On the other hand, the difference between *T. evansi* and *T. brucei*, which lies in the kinetoplast DNA (kDNA), has been also well documented. The kDNA of *T. brucei* consists of 50-100 homogeneous maxicircles and 5,000-10,000 heterogeneous minicircles (Borst and Hoeijmakers 1979). The maxicircle DNA resembles a conventional mitochondrial DNA in which it encodes ribosomal RNA and a small number of proteins. While, the minicircle DNA encodes guide RNA which plays an important role for post-transcriptional editing of maxicircle DNA transcripts (Benne et al. 1986; Bhat et al. 1990; van der Spek et al. 1990). In contrast, *T. evansi* lacks maxicircle DNA (Borst et al. 1979), and its minicircle DNAs of *T. evansi* are highly homogeneous and possess the same, or nearly the same, DNA sequences (Songa et al. 1990; Ou, Giroud and Baltz. 1991). It was further reported that several features of the sequence of minicircle DNAs were specific for *T. evansi*, and that such sequences would be of useful in distinguishing *T. evansi* from *T. brucei* by means of DNA hybridization technique and polymerase chain reaction (PCR) (Masiga et al. 1990; Artama et al. 1992). However, it is necessary to consider wide prevalence of akinetoplastic strains of *T. evansi* when such a *T. evansi* specific minicircle DNA sequence is used as a diagnostic tool. Moreover, if origin(s) of *T. evansi* was *T. brucei* spp., it would be possible that certain strains of *T. brucei* might have the *T. evansi* specific minicircle DNA sequence in their heterogeneous minicircle DNA. In this study, we examined such a possibility in various strains of *T. brucei*, including of *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*.

Procyclic acidic repetitive protein (PARP) is a major surface glycoprotein of procyclic form of *T. brucei* (Mowatt et al. 1987; Richardson et al. 1988).

Although the function of the PARP gene is not fully understood, it may play a role in the protection of the parasite against proteases present in the midgut of tsetse vectors (*Glossina* spp.) (Roditi and Pearson. 1990). The PARP expression in bloodstream forms is down-regulated relative to procyclic forms at several levels (Hotz et al. 1998). However, a relatively high level of transcription occurs from the procyclin promoter in the bloodstream forms (Pays et al. 1990; Vanhamme et al. 1995; Vanhamme and Pays 1998). In consideration with life cycle of *T. evansi*, the PARP seems to be unnecessary in *T. evansi*. However, interestingly, the PARP A-α gene was found by PCR-based analyses of *T. evansi* (Artama et al. 1992). In this study, potential use of the PARP primers as a diagnostic tool for *T. evansi* was explored, specially its application outside of the tsetse belt in where both of the kinetoplastic and the akinetoplastic strains of *T. evansi* are known to be widely prevalent but not *T. brucei*. Moreover, further characterization of the PARP A-α gene of *T. evansi* was made regarding its gene expression in *T. evansi*.

MATERIALS AND METHODS

Trypanosomes:

Histories of the strains of *T. evansi* and those of *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense* and *T. congolense* are listed in Tables 1 and 2, respectively. The bloodstream forms of trypanosomes were propagated in normal, semi-lethaly irradiated (600 rad) or severe combined immunodeficient (SCID) mice (Inoue et al. 1998), and separated from the blood cells by means of DE-52 cellulose column chromatography (Lanham and Godfrey 1970). The procyclic forms of *T. b. rhodesiense* IL2343 were produced in vitro following the procedure by Hirumi et al. (1991).

Table 1. History of *Trypanosoma evansi* strains tested

Strain	Place	Year	Host	Tsetse
IL1695	Kenya	1978	Camel	Negative
IL1934	South America	1971	Capybara	Negative
IL3354	Mali	1988	Camel	Negative
IL3382	Mali	1988	Camel	ND
IL3960	Kenya	1980	Camel	Negative
IL3962	Sudan	1976	Camel	Negative
Tansui	Taiwan	ND	Waterbuffalo	ND

Place: Place of isolation, Year: Year of isolation, Tsetse: tsetse transmissibility, ND: No data, Except for data of Tansui, all information was obtained from ILRI's Biological Service Unit.

Extraction of DNA:

Kinetoplast DNA was extracted from the trypanosomes as described by Waitumbi and Young (1994) with minor modifications. Briefly, isolated trypanosomes (>10^7) were incubated in lysing buffer (1% SDS, 100 μg/ml proteinase K, 0.2 M NaCl, 10 mM Tris-HCl, 10 mM EDTA, pH 8.0) at 50°C for 24 hrs. The genomic DNA was sheared by repeatedly passing the lysate through a 27G needle, and kDNA was pelleted by centrifugation at 12,000*g* at 20°C for 30 min. Kinetoplast DNA networks were washed 3 times in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The purified kDNA networks were stored in TE buffer at 4°C up to use. Genomic DNA was extracted as described elsewhere (Kaiser, Murray and Whittaker 1995).

Table 2. History of *Trypanosoma* strains tested

Species	Strain	Ref. Stock	Place	Year
T. b. brucei	GUTat3.1	ND	Uganda	1966
T. b. brucei	221	427	ND	ND
T. b. gambiense	IL1922	ILTat1.1	Ivory Coast	1952
T. b. gambiense	IL3248	IL3248	Nigeria	1969
T. b. gambiense	IL3250	IL3250	Nigeria	1969
T. b. gambiense	IL3253	T.g.2565	South Sudan	1982
T. b. gambiense	IL3254	T.g.2571	South Sudan	1982
T. b. gambiense	IL3301	IL3249	Nigeria	1969
T. b. gambiense	IL3707	IL3247	Nigeria	1968
T. b. gambiense	Welcome	ND	ND	ND
T. b. rhodesiense	IL1501	KETRI 2503	Kenya	1980
T. b. rhodesiense	IL2343*	STIB386AA	Ivory Coast	1978
T. congolense	IL3000	Trans Mara I	Kenya/Tanzania border	1966

Ref. Stock: Reference stock, Place: Place of isolation, Year: Year of isolation, ND: no data, *: IL2343 was originally isolated from a 'gambiense' sleeping sickness patient but was later classified as *T. b. rhodesiense* (Hide et al. 1990).

DNA cloning and sequencing:

The kDNA networks of *T. evansi* was digested with *Taq* I and the fragment separated by means of high performance liquid chromatography (TSKgel DNA-NPR, TOSO, Japan). The fragment ends were converted to blunt by using klenow fragment. Then the fragments were ligated into the unique *Sma* I site of pUC 19

plasmids (Kaiser, Murray and Whittaker 1995). The cloned fragments of kDNA were sequenced by means of dye primer cycle sequencing method (ABI PRISM™ Dye Primer Cycle Sequencing Ready Reaction Kit, Cat. No. 402113, The Perkin-Elmer Co., U.S.A). The PCR products from genomic DNA of trypanosomes (described below) were sequenced directly.

Polymerase chain reaction (PCR):

PCR amplification of minicircle DNA and PARP A-α gene were performed as described (Artama et al. 1992). For the PCR amplification of minicircle DNA, PARP A gene and PARP core promoter, the following oligonucleotide primer sets were used.

Minicircle primers:
5'-CAA CGA CAA AGA GTC AGT-3' and 5'-ACG TGT TTT GTG TAT GGT-3'

PARP A gene primers:
5'-CAC AAT GGC ACC TCG TTC CC-3' and 5'-TTA GAA TGC GGC AAC GAG A-3'

PARP core promoter primers:
5'-GCA GTC AGC CTT TGT TGT CA-3' and 5'-TTA GAA TGC GGC AAC GAG A-3'

The amplifications were conducted for 30 cycles in a DNA Thermal Cycler (The Perkin-Elmer Co., U.S.A.) in which each cycle carried out by successive incubations of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec.

Reverse transcription PCR (RT-PCR):

Total RNA was extracted by an acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987). To delete contamination of genomic DNA, 10 μg of the total RNA was treated with 200 units of deoxyribonuclease I (Takara Shuzo Co. Ltd., Japan). Oligo-dT primed cDNA was synthesized by using kit (Ready-To-Go™ You-Prime First-Strand Beads, Pharmacia Biotech Ltd., U.S.A.). PCR amplifications of cDNA were conducted in a 50 μl reaction mixture containing 1 μl cDNA reaction mixture, 0.5 μM each of primers, 2.5 units of *Taq* DNA polymerase (The Perkin-Elmer Co., U.S.A.), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dATP, dGTP, dCTP and dTTP. The oligonucleotide primers and the temperature conditions for the amplification were the same as described above.

RESULTS

Cloning and Sequencing of minicircle DNA:

Firstly, the determination of the complete sequences of minicircle DNA of all the *T. evansi* strains were made, except for akinetoplastic *T. evansi* IL1934. All minicircle DNA sequences were aligned from the GGGGTTGGTGTA sequence

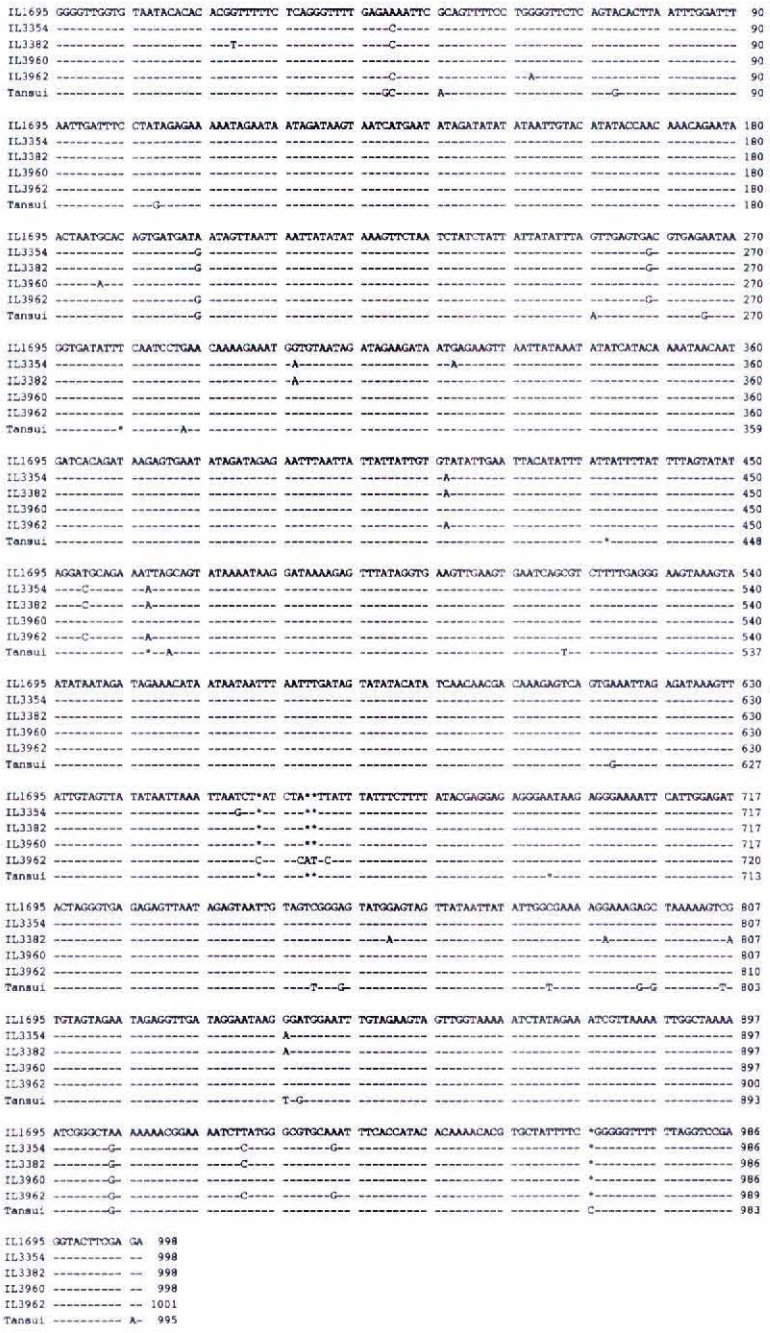

Figure 1. Alignments of the entire sequences of the minicircle DNA from *Trypanosoma evansi* strains IL1695, IL3354, IL3382, IL3960, IL3962 and Tansui. Nucleotides identical to those in IL1695 sequence are indicated by dashes (-). Deletions are marked by asterisks (*).

which is the putative replication origin of minicircle DNA (Birkenmeyer, Sugisaki and Ray 1987; Ntambi et al. 1986) (Fig. 1). Minicircle DNA sequence was highly conserved among *T. evansi* strains which isolated in different countries or places. Attempts to isolate the kDNA networks of the akinetoplastic *T. evansi* IL1934 were made several times but were vain as expected. Sequence homology was calculated among newly determined 6 minicircle DNA sequences and 4 previously reported (Ou et al. 1991) complete minicircle DNA sequences of *T. evansi* (Table 3). The homology was more than 96% among all the samples tested.

Table 3. Pairwise analysis of sequence homology (%) among the minicircle DNA from *Trypanosoma evansi* strains.

	IL1695	IL3354	IL3382	IL3960	IL3962	Tansui	MA1*	MA2*	ET*	SH*
IL1695		98	98	99	98	97	97	97	98	97
IL3354			99	98	99	96	96	96	98	96
IL3382				98	98	96	96	96	98	97
IL3960					98	97	97	97	98	97
IL3962						96	96	96	97	96
Tansui							97	96	97	97
MA1*								99	98	98
MA2*									98	98
ET*										97

*Ou et al. 1991.

Secondly, possible existence of the "*T. evansi* specific" minicircle DNA in the kDNA networks of various strains of *T. brucei* was examined. Total DNA and/or kDNA were prepared from 7 strains of *T. evansi* (IL1695, IL1934, IL3354, IL3382, IL3960, IL3962 and Tansui), 2 strains of *T. b. brucei* (GUTat3.1 and 221), 8 strains of *T. b. gambiense* (IL1922, IL3248, IL3250, IL3253, IL3254, IL3301, IL3707 and Welcome), 2 strains of *T. b. rhodesiense* (IL1501 and IL2343) and *T. congolense* IL3000. And then, minicircle DNA was amplified by means of "*T. evansi* minicircle DNA specific PCR" (Artama et al. 1992). Positive reaction was obtained from total DNA and/or purified kDNA of 6 strains of *T. evansi* (IL1695, IL3354, IL3382, IL3960, IL3962 and Tansui), *T. b. brucei* GUTat3.1, *T. b. gambiense* Welcome and 2 strains of *T. b. rhodesiense* (IL1501 and IL2343). Nucleotide sequence of these PCR products were determined and the sequence homology was calculated among them (Table 4). The homology was more than 93% among *T. evansi* strains, *T. b. gambiense* Welcome and *T. b.*

rhodesiense IL1501. While, the homology was less than 62% among *T. evansi* strains, *T. b. brucei* GUTat3.1 and *T. b. rhodesiense* IL2343.

Table 4. Pairwise analysis of sequence homology (%) among the minicircle DNA fragments from *Trypanosoma evansi* and *T. brucei*.

	GUTat 3.1	Welcome	IL1501	IL2343
IL1695	61	99	94	24
IL3354	61	98	95	24
IL3382	61	98	93	24
IL3960	61	100	95	24
IL3962	61	99	95	24
Tansui	62	97	94	24
GUTat 3.1		37	37	12
Welcome			95	24
IL1501				24

```
427[1)]   MAPRS LYLLA ILLFS ANLFA GVGFA AAADE SASNV IVKGG KGKER EDGPE  50
221       ----- ----- ----- ----- ----- ----- ----- ----- ----- -----  50
Welcome   ----- ----- ----- ----- ----- -T--- ----- ----- ----- -----  50
IL2343    ----- ----- V---- ----- ----- ----- ----- ----- ----- -----  50
IL1695    ----- ----- ----- ----- ----- ----- ----- ----- ----- -----  50
IL1934    ----- ----- ----- ----- ----- ----- ----- ----- ----- -----  50
IL3354    ----- ----- V---- ----- ----- ----- ----- ----- ----- -----  50
IL3382    ----- ----- V---- ----- ----- ----- ----- ----- ----- -----  50
IL3960    ----- ----- ----- ----- ----- ----- ----- ----- ----- -----  50

427[1)]   EPEET GPEET GPEET GPEET GPEET GPEET GPEET EPEPE PGAAT LKSVA 100
221       ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- 100
Welcome   ----- ----- ----- ----- ----- ----- ***** ----- ----- -----  95
IL2343    ----- ----- ----- ----- ----- ----- ***** ----- ----- -----  95
IL1695    ----- ----- ----- ----- ----- ----- ***** ----- ----- -----  95
IL1934    ----- ----- ----- ----- ----- ***** ***** ----- ----- -----  90
IL3354    ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- 100
IL3382    ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- 100
IL3960    ----- ----- ----- ----- ----- ----- ***** ----- ----- -----  95
```

Figure 2. Alignments of the predictive amino acid sequences of the PARP A-α (M1–A100) from *Trypanosoma evansi* (IL1695, IL1934, IL3354, IL3382 and IL3960) and *T. brucei* (427, 221, Welcome and IL2343) strains. Amino acids identical to those in 427 sequence are indicated by dashes (-). Deletions are marked by asterisks (*). 1)Mowatt, Wisdom and Clayton 1989.

Sequence analyses of PARP A-α gene:

Procyclic acidic repetitive protein gene was amplified by PCR from genomic DNA of 5 strains of *T. evansi* (IL1695, IL1934, IL3354, IL3382 and

IL3960), *T. b. brucei* 221, *T. b. gambiense* Welcome and *T. b. rhodesiense* IL2343. Then, the determinations of the DNA sequence of all the PCR products were made in order to compare PARP A gene of *T. evansi* with that of *T. brucei*. Since the PCR was targeted to the open reading frame of the PARP A-α gene, nucleotide sequence had been translated to amino acid sequence. Then, the amino acid sequences of the 5 strains of *T. evansi*, *T. b. brucei* 221, *T. b. gambiense* Welcome and *T. b. rhodesiense* IL2343 were aligned (Fig. 2). Except for the number of GPEET repeat, the amino acid sequence was (nearly) identical among *T. evansi* and *T. brucei* strains tested.

```
IL1695  GCAGTCAGCCTTTGTTGTCATTGGGGTTAAGCGGAAAGGTGTGTGTCAGT  50
IL2343  GCAGTCAGCCTTTGTTGTCATTGGGGTTAGGTGGGAAGGTGTGTGCCAGT  50

IL1695  AGGTTGTGAGGTGAAAGCGTTTTCAGATGCATAGTGAGCTTAATGTCCTT 100
IL2343  AGGTTGTGAGGTGAAAGCGTTTTCAGATGCATAGTGAGCTTAATGTCCTT 100

IL1695  TTCACAGTATATCGTGTCTGATAGGTATCTCTTATTAGTATAGTCGAATA 150
IL2343  TTCACAGTATATCGTGTCTGATAGGTATCTCTTATTAGTATAGTCGAATA 150

IL1695  CTAGTCAATAGTGCGTTTTGTGCAAAATGTCCATTTTGTGGCAGTGATGG 200
IL2343  CTAGTCAATAGTGCATTTTGTGCAAAATGTCCATTTTGTGGCAGTGATGG 200

IL1695  GGTTGTTTTATGCTATTCCGTGTCTCTGGGTGGGCGTGCATTGAAAATAG 250
IL2343  GGTTGTTTTGTGCTGTTCCGTGTCTCTGGGTGGGCGTGCATTGAAAATAG 250

IL1695  GGGTTATCGGGTGAGTACTGAGTTTAACATGTTCTCGTGATCGCTGCACG 300
IL2343  GGGTTATTAGGTGAGTACTGAGTTTAAGATGTTCTCGTGATCGCTGCACG 300

IL1695  CGCCTTCGGGTTTTTTTT*CCTTTTCCCCATTTTTTTCAACTTGAAGACT 349
IL2343  CGCCTTCGAGTTTTTTTTTCCTTTTACCCATTTTTTTCAACTTGAAGACT 350

IL1695  TCAATTACACCAAAAAGTAAAATTCACAATGGCACCTCGTTCCCTTTATC 399
IL2343  TCAATTACACCAAAAAGTAAAATTCACAATGGCACCTCGTTCCCTTTATC 400
                                    M  A  P  R  S  L  Y  L

IL1695  TGCTCGCTATTCTTCTGTTCAGCGCGAACCTCTTCGCTGGCGTGGGATTT 449
IL2343  TGCTCGCTGTTCTTCTGTTCAGCGCGAACCTCTTCGCTGGCGTGGGATTT 450
          L  A  I  L  L  F  S  A  N  L  F  A  G  V  G  F
                V
IL1695  GCCGCAGCCGCTGATGAGTCGGCTAGCAACGTTATCGTGAAGGGAGGCAA 499
IL2343  GCCGCAGCCGCTGATGAGTCGGCTAGCAACGTTATCGTGAAGGGAGGCAA 500
        A  A  A  A  D  E  S  A  S  N  V  I  V  K  G  G  K

IL1695  AGGAAAGGAGAGGGAGGACGGCCCTGAGGAGCCGGAAGAGACCGGACCAG 549
IL2343  AGGAAAGGAGAGGGAGGACGGCCCTGAGGAGCCGGAAGAAACCGGACCAG 550
         G  K  E  R  E  D  G  P  E  E  P  E  E  T  G  P  E

IL1695  AAGAGACCGGACCAGAAGAAACCGGACCAGAAGAGACGGGGCCGGAAGAG 599
IL2343  AAGAGACCGGACCAGAAGAAACCGGACCAGAAGAGACGGGGCCGGAAGAG 600
          E  T  G  P  E  E  T  G  P  E  E  T  G  P  E  E

IL1695  ACGGGACCAGAGGAAACTGAACCTGAACCTGAACCTGGTGCTGCAACGCT 649
IL2343  ACGGGACCAGAGGAAACTGAACCTGAACCTGAACCTGGTGCTGCAACGCT 650
        T  G  P  E  E  T  E  P  E  P  E  P  G  A  A  T  L

IL1695  GAAATCTGTTGCACTTCCGTTTGCAGTCGCGGCTGCTGCTCTCGTTGCCG 699
IL2343  GAAATCTGTTGCACTTCCGTTTGCAGTCGCGGCTGCTGCTCTCGTTGCCG 700
         K  S  V  A  L  P  F  A  V  A  A  A  A  L  V  A  A

IL1695  CATTCTAA                                          713
IL2343  CATTCTAA                                          709
          F
```

Figure 3. Comparison of the nucleotide sequences of a PARP core promoter (boxed sequences) from *Trypanosoma evansi* IL1695 and *T. brucei rhodesiense* IL2343. Arrows show the sequences of sense and anti-sense primers for the PCR amplification. Deletion is marked by asterisks (*). The predictive amino acid sequences shown under the nucleotide sequences are the entire sequence of the PARP A-α.

A PARP promoter is one of the well known promoter in *T. brucei* (Brown, Huang and van der Ploeg 1992; Sherman et al. 1991). Oligonucleotide primer pair was designed to amplify from the PARP core promoter region to the end of the open reading frame of the PARP A-α. The PCR products were cloned and sequenced. The PARP core promoter sequences of *T. evansi* IL1695 and *T. b. rhodesiense* IL2343 were the same (Fig. 3).

The PARP A-α gene expression in T. evansi:

Total RNA was obtained from procyclic forms and bloodstream forms of *T. b. rhodesiense* IL2343, and *T. evansi* IL1695. The oligo-dT primed cDNA was employed as a template for PCR. Regions of the PARP A gene primers and the PARP core promoter primers are illustrated in Figure 4. Since one of the PARP core promoter primer region was out of the transcription initiation site, this primer pair helped to discriminate between positive reaction caused by genomic DNA contamination and real positive reaction. The results are shown in the Figure 5. The RT-PCR by using the PARP A gene primer were positive in the all the cDNA samples, and the same amplification patterns were observed in the genomic DNA samples. Expectedly, there were no amplification in the cDNA samples with the PARP core promoter primer.

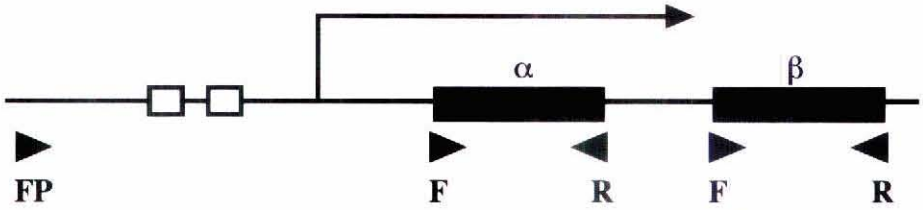

Figure 4. Map of the PARP A gene showing the positions of the binding sites (arrow head) for the PARP gene primers (F and R) and the PARP core promoter primers (FP and R). Transcription is schematically represented by arrowed line. Closed boxes indicate open reading frames of the PARP A-α (α) and the PARP A-β (β). Open boxes indicate the PARP core promoter region.

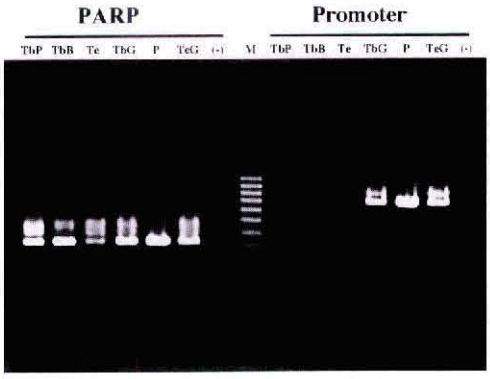

Figure 5. The RT-PCR amplification patterns of primer pairs specific for PARP gene (PARP) and PARP core promoter (Promoter) of *Trypanosoma brucei*, and template cDNA of *T. b. rhodesiense* IL2343 procyclic forms (TbP), *T. b. rhodesiense* IL2343 bloodstream forms (TbB) and *T. evansi* IL1695 (Te). Lane TbG, P and TeG are *T. b. rhodesiense* IL2343 genomic DNA, the PARP A-α gene cloned into pUC19 plasmid and *T. evansi* IL1695 genomic DNA respectively. Lane (-) is negative control without template DNA, and lane M is 100 bp ladder.

Evaluation of the specificity of the PARP A gene primers:

To assess a potential use of the PARP A gene primer as a diagnostic tool for *T. evansi* and *T. brucei* infection, the PCR was performed by using various species of protozoan parasites including *Trypanosoma*, *Babesia*, *Neospora and Toxoplasma*. Genomic DNA was extracted form 7 strains of *T. evansi* (IL1695, IL1934, IL3354, IL3382, IL3960, IL3962 and Tansui), 10 strains of *T. brucei* (IL1922, IL3248, IL3250, IL3253, IL3254, IL3301, IL3707, Welcome, IL1501 and GUTat3.1), *T. congolense* IL3000, *Babesia equi* USDA strain and *B. caballi* USDA strain (a gift of Dr. Ikadai, The Research Center for Protozoan Molecular Immunology, Obihiro University, Japan), *Neospora caninum* NC-1 strain (a gift of Dr. Dubey, USDA, USA) and *Toxoplasma gondii* RH strain (a gift of Dr. Nishikawa, The Research Center for Protozoan Molecular Immunology, Obihiro University, Japan), and used as templates for the PCR. As a result, the same amplification patterns were obtained from all *T. brucei* and *T. evansi* strains tested. While the PCR was negative in the other species tested (Fig. 6).

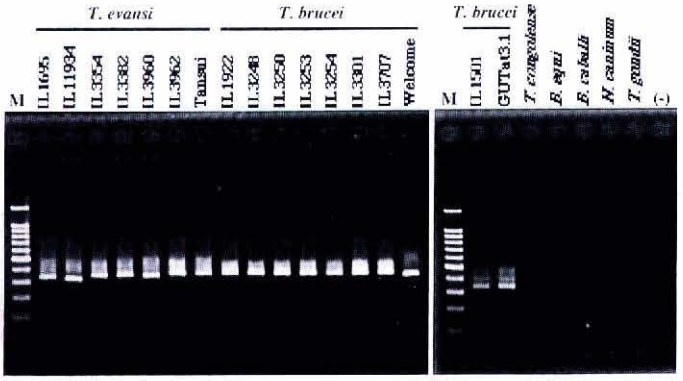

Figure 6. The PCR amplification patterns of primer pairs specific for PARP gene among several species of protozoan parasites. Lane M is 100 bp ladder, and lane (-) is negative control without template DNA.

DISCUSSION

In the previous studies, the kDNA minicircle of *T. evansi* was characterized by restriction enzyme digestion patterns, and partial and total sequencing (Borst, Fase-Fowler and Gibson 1987; Songa et al. 1990; Ou et al. 1991). According to the analyses of restriction enzyme digestion patterns of kDNA minicircles (Borst et al. 1987; Songa et al. 1990), *T. evansi* kDNA minicircles have been classified into 4 closely related types (A1, A2, A3 and A4) and one different type (B1). In this study, we have determined total sequence of kDNA minicircles of 6 *T. evansi* strains isolated from several countries or places of Africa, and Taiwan (Fig. 1). It was reported that the kDNA minicircle of *T. evansi* IL1695 belonged to the type A1 minicircle (Borst et al. 1987). The results of the total sequencing obtained in this study showed that the five strains belonged to the type A1 or A2 minicircle

(A1: IL1695, IL3382, IL3960 and IL3962, A2: IL3354), and to one new related type (Tansui). Total sequences of kDNA minicircle of *T. evansi* strains (NJ and SH: China, MA: The Philippines, ET: Ethiopia) also reported by Ou et al. (1991). We calculated the sequence homology among 10 *T. evansi* strains including 4 previously reported sequences (Table. 3). The results clearly indicated that the kDNA minicircle of *T. evansi* is highly conserved among the strains isolated from several countries or places of Africa and Asia.

It has been suggested that *T. evansi* was "recently" evolved from *T. brucei.* Moreover, it was reported that putative guide RNA sequences were coded in minicircle DNA of *T. evansi* (Gajendran et al. 1992). Therefore, we inferred that *T. evansi* type kDNA minicircle might exist in several strains of *T. brucei.* To elucidate this speculation, total DNA and/or purified kDNA networks from various strains of *T. brucei* and a strain of *T. congolense* were examined by means of "*T. evansi* minicircle specific PCR" (Artama et al. 1992). The result revealed that *T. b. gambiense* Welcome strain and *T. b. rhodesiense* IL1501 also possess *T. evansi* type minicircle DNA (Table 4). This suggests that the minicircle DNA of *T. evansi* has a potential for a source of guide RNA (although it might be not functional) which might be unnecessary in *T. evansi*, and that the two *T. brucei* strains seem to be in a closed relation with *T. evansi* in terms of minicircle DNA.

Comparative study of the PARP A-α gene sequences among 4 strains of *T. brucei* and 5 strains of *T. evansi* revealed that the PARP A-α gene of *T. evansi* is highly conserved. A predictive amino acid sequence of the PARP A-α in *T. evansi* and *T. brucei* were (nearly) the same (Fig. 2). We also examined nucleotide sequences of a PARP core promoter region of *T. evansi* IL1695. Interestingly, the PARP core promoter sequence of *T. evansi* IL1695 and *T. b. rhodesiense* IL2343 were the same (Fig. 3). The PARP expression in bloodstream forms is down-regulated relative to procyclic forms. However, it has been demonstrated that the PARP promoter is active in bloodstream forms (Pays et al. 1990; Vanhamme et al. 1995, 1998). Therefore, we inferred that the PARP gene expressed in *T. evansi.* The RT-PCR amplification patterns clearly indicated that the PARP gene expresses in the bloodstream form not only of *T. brucei* but also of *T. evansi* (Fig. 5). The several bands in each lane are caused by different PARP genes. The PARP expression has been shown to be up-regulated by differentiation triggering factors such as low temperature and mild acid stress (Pays et al. 1990; Rolin et al. 1998). Since *T. evansi* survive for a few days in its insect vector (Woo 1977), the PARP expression may be up-regulated during this period, although due to the absence of maxicircle DNA and the loss of minicircle DNA heterogeneity, *T. evansi* may not be able to differentiate into the insect form.

In conclusion, this study also strongly indicated that *T. evansi* was

"recently" evolved from *T. brucei* on the bases of (I) the finding of the *T. brucei* strains which have "*T. evansi* specific" minicircle DNA sequences in their kDNA networks, (II) a further evidence that the PARP gene is not only conserved but also expressed in *T. evansi*, in addition to the previous findings (Gibson et al. 1983; Masiga et al. 1990; Stevens et al. 1992). Until now, it is not clear if the impairment of kDNA is the only reason why *T. evansi* do not have developmental stages in its insect vector. Comparative study of responses of *T. brucei* and *T. evansi* to differentiation triggering factors may clarify the molecular mechanisms that underlie the differentiation during the life cycle of *T. brucei*.

Although "*T. evansi* specific" minicircle DNA primer is a useful tool for diagnosis of *T. evansi* (Artama et al. 1992), it may overlook akinetoplastic strains in where such strains, such as *T. evansi* IL1934, are plevarent. The finding of the "*T. evansi* type" minicircle DNA sequence in 2 strains of *T. brucei* also suggest need of precaution in diagnosing *T. evansi* infection particularly in areas where a critical distinction of *T. evansi* and *T. brucei* infections is required.

Although the PCR detection using the PARP gene primers is also unable to distinct *T. evansi* infection from that of *T. brucei*, it detects the infection with akinetoplastic strains of *T. evansi*. Therefore, the PARP gene primers may be a useful tool for the diagnosis of *T. evansi* in the tsetse-free regions, and may be also used inside of the tsetse belt if the distinction between *T. evansi* and *T. brucei* infections are not so critical.

ACKNOWLEDEMENTS

We thank Dr. T. Fukuma (Dept. of Parasitology, Kurume Univ., School of Medicine) for providing *T. b. rhodesiense* IL1501, Dr. M. Horio (Dept. of Parasitology and Tropical Public Health, Univ. of Occupational and Environmental Health, School of Medicine) for *T. b. gambiense* Welcome, Dr. T. Nozaki (Dept. of Tropical Medicine and Parasitology, School of Medicine, Keio Univ.) for *T. b. brucei* 221 and Dr. T. Yanagi (Dept. of Protozoology, Institute of Tropical Medicine, Nagasaki Univ.) for *T. evansi* Tansui. This study is supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Encouragement of Young Scientists 09760263.

REFERENCES

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

Benne, R., van den Burg, J., Brakenhoff, J. P. J., Sloof, P., van Boom, J. H. &

Tromp, M. C. 1986. Major transcript of the frameshifted *cox* II gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell 46:* 819-826.

Bhat, G. J., Koslowsky, D. J., Feagin, J. E., Smiley, B. L. & Stuart, K. 1990. An extensively edited mitochondrial transcript in kinetoplastids encodes a protein homologous to ATPase subunit 6. *Cell 61:* 885-894.

Birkenmeyer, L., Sugisaki, H. & Ray, D. S. 1987. Structural characterization of site-specific discontinuities associated with replication origins of minicircle DNA from *Crithidia fasciculata*. *J. Biol. Chem. 262:* 2384-2392.

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

Borst, P. & Hoeijmakers, J. H. J. 1979. Kinetoplast DNA. *Plasmid 2:* 20-40.

Brown, S. D., Huang, J. & van der Ploeg, L. H. 1992. The promoter for the procyclic acidic repetitive protein (PARP) genes of *Trypanosoma brucei* shares features with RNA polymerase I promoters. *Mol. Cell Biol. 12:* 2644-2652.

Chomczynski, P. & Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem. 162:* 156-159.

Gajendran, N., Vanhecke, D., Songa, E. B. & Hamers, R. 1992. Kinetoplast minicircle DNA of *Trypanosoma evansi* encode guide RNA genes. *Nucleic Acids Res. 20:* 614.

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

Hide, G., Cattand, P., LeRay, D., Barry, D. & Tait, A. 1990. The identification of *Trypanosoma brucei* subspecies using repetitive DNA sequences. *Mol. Biochem. Parasitol. 39:* 213-226.

Hirumi, H., Hirumi, K., Moloo, S. K. & Shaw, M. K. 1991. In vitro cultivation of blood stream trypomastigotes of *Trypanosoma vivax* without feeder cell layers. *J. Protozool. Res. 1:* 1-12.

Hotz, H-R., Biebinger, S., Flaspohler, J. & Clayton, C. 1998. PARP gene expression: control at many levels. *Mol. Biochem. Parasitol. 91:* 131-143.

Inoue, N., Narumi, D., Mbati, P. A., Hirumi, K., Situakibanza, N-T. H. & Hirumi, H. 1998. Susceptibility of severe combined immuno-deficient (SCID) mice to *Trypanosoma brucei gambiense* and *T. b. rhodesiense*. *Trop. Med. Int. Health 3:* 408-412.

Kaiser, K., Murray, N. E. & Whittaker, P. A. 1995. Construction of representative genomic DNA libraries using phage lambda replacement vectors. pp. 37-84.

In: DNA Cloning 1, Glover, D. M. & Hames, B. D. (Ed), IRL Press, Oxford.

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

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

Mowatt, M. R. & Clayton C. E. 1987. Developmental regulation of a novel repetitive protein of *Trypanosoma brucei. Mol. Cell Biol. 7:* 2838-2844.

Mowatt, M. R., Wisdom, G. S. & Clayton, C. E. 1989. Variation of tandem repeats in the developmentally regulated procyclic acidic repetitive proteins of *Trypanosoma brucei. Mol. Cell Biol. 9:* 1332-1335.

Ntambi, J. M., Shapiro, T. A., Ryan, K. A. & Englund, P. T. 1986. Ribonucleotides associated with a gap in newly replicated kinetoplast DNA minicircles from *Trypanosoma equiperdum. J. Biol. Chem. 261:* 11890-11895.

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

Pays, E., Coquelet, H., Tebabi, P., Pays, A., Jefferies, D., Steinert, P., Koenig, E., Williams, R. O. & Roditi, I. 1990. *Trypanosoma brucei*: constitutive activity of the VSG and procyclin gene promoters. *EMBO J. 9:* 3145-3151.

Richardson, J. P., Beecroft, R. P., Tolson, D. L., Liu, M. K. & Pearson, T. W. 1988. Procyclin: an unusual immunodominant glycoprotein surface antigen from the procyclic stage of African trypanosomes. *Mol. Biochem. Parasitol. 31:* 203-216.

Roditi, I. & Pearson, T. W. 1990. The procyclin coat of African trypanosomes. *Parasitology Today 6:* 79-82.

Rolin, S., Hanocq-Quertier, J., Paturiaux-Hanocq, F., Nolan, D. P. & Pays, E. 1998. Mild acid stress as a differentiation trigger in *Trypanosoma brucei. Mol. Biochem. Parasitol. 93:* 251-262.

Sherman, D. R., Janz, L., Hug, M. & Clayton, C. 1991. Anatomy of the parp gene promoter of *Trypanosoma brucei. EMBO J. 10:* 3379-3386.

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.

Stevens, J. R., Lanham, S. M., Allingham, R. & Gashumba, J. K. 1992. A simplified method for identifying subspecies and strain groups in *Trypanozoon* by isoenzymes. *Ann. Trop. Med. Parasitol. 86:* 9-28.

Stuart, K. 1983. Kinetoplast DNA, mitochondrial DNA with a difference. *Mol. Biochem. Parasitol. 9:* 93-104.

van der Spek, H., Speijer, D., Arts, G-J., van den Burg, J., van Steeg, H., Sloof, P. & Benne, R. 1990. RNA editing in transcripts of the mitochondrial genes of the insect trypanosome *Crithidia fasciculata*. *EMBO J. 9:* 257-262.

Vanhamme, L., Berberof, M., Le Ray, D. & Pays, E. 1995. Stimuli of differentiation regulate RNA elongation in the transcription units for the major stage-specific antigens of *Trypanosoma brucei*. *Nucleic Acids Res. 23:* 1862-1869.

Vanhamme, L. & Pays, E. 1998. Controls of the expression of the Vsg in *Trypanosoma brucei*. *Mol. Biochem. Parasitol. 91:* 107-116.

Waitumbi, J. N. & Young , J. R. 1994. Electrophoretic karyotyping is a sensitive epidemiological tool for studying *Trypanosoma evansi* infections. *Vet. Parasitol. 52:* 47-56.

Woo, P. *T.* K. 1977. Salivarian trypanosomes producing disease in livestock outside of sub-saharan Africa. pp. 273-275. *In:* Parasitic Protozoa, Volume I, Kreier, J. P. (Ed), Academic Press, Inc., New York.