

KINETOPLAST DNA AND RNA OF *TRYPANOSOMA BRUCEI*

AGDA M. SIMPSON and LARRY SIMPSON

Biology Department and Molecular Biology Institute, University of California, Los Angeles, CA 90024, U.S.A.

(Received 26 February 1980; accepted 23 May 1980)

Kinetoplast DNA (kDNA) and kinetoplast RNA (kRNA) were isolated from bloodstream and procyclic culture forms of two clonal strains of *Trypanosoma brucei*. No differences were observed in kDNA (maxicircle) restriction profiles between bloodstream or procyclic culture forms of the same strain. Some differences were observed in kDNA maxicircle restriction sites between the two strains. Buoyant density analysis of Pst I digested kDNA showed the release of a minor low density band representing unit length linearized maxicircle DNA. Pst I or Bam H1-linearized maxicircle DNA was isolated by the Hoechst 33258 dye–CsCl method and a restriction enzyme map of the maxicircle was constructed. Closed monomeric minicircles released from kDNA networks by sonication sedimented with a molecular size of around 1100 base pairs. A substantial minor length heterogeneity was evident in acrylamide gel electrophoresis of once cut minicircles. Several minicircle sequence classes and two Hind III maxicircle fragments representing approx. 50% of the maxicircle were cloned in the bacterial plasmid, pBR322, in *Escherichia coli*. A purified kinetoplast-mitochondrion fraction was isolated from procyclic culture forms by the Renografin flotation method. The major kRNA components were two small RNAs which comigrated with *Leishmania tarentolae* 9 and 12 S kRNAs in denaturing gels. These RNAs hybridized to the maxicircle component of the kDNA, specifically to the smaller cloned Hind III maxicircle fragment. This cloned fragment had substantial sequence homology with the cloned maxicircle fragment from *L. tarentolae* which contains the 9 and 12 S RNA genes, implying an evolutionary conservation of the 9 and 12 S gene sequences. Identical kRNAs were observed in cultured bloodstream forms of *T. brucei*.

Key words: *Trypanosoma brucei*, Kinetoplast DNA, Kinetoplast RNA, Recombinant DNA, Maxicircle DNA.

INTRODUCTION

The hemoflagellate protozoa contain an unusual mitochondrial DNA known as kinetoplast DNA (kDNA) [1, 2]. This DNA consists of a single large network of catenated minicircles and maxicircles. kDNA has been isolated from several species belonging to the genera *Crithidia* [3–6], *Leishmania* [7, 8], *Phytomonas* [9] and *Trypanosoma* [10]. Apparent phylogenetic changes can be seen in the size and complexity of the minicircle

Abbreviations: EthBr, ethidium bromide; kDNA, kinetoplast DNA; kRNA, kinetoplast RNA; kb, kilobase pairs; SSC, saline-sodium citrate solution.

component and in the size of the maxicircle component, with the presumably more primitive species possessing larger, less complex minicircle DNA, and the more advanced species possessing smaller, more complex minicircle DNA; the maxicircle DNA on the other hand is generally larger in the more primitive species and smaller in the more advanced species (with the exception of *Trypanosoma cruzi* where the maxicircle is relatively large [11]).

The kDNA of the trypanosomes belonging to the *Trypanosoma brucei* group represents an extreme in terms of the minicircle complexity and the size of the maxicircle DNA. The minicircles, which are approx. 1000 nucleotides in size, are present in approx. 200 semihomologous sequence classes [12], two of which have been recently cloned and sequenced [13]. The maxicircle DNA is approx. 20–22 kilobase pairs (kb) in size, and restriction maps of maxicircle DNA from two strains of *T. brucei* [14, 15] and from one strain of the closely related species, *Trypanosoma equiperdum* [16] have recently been reported. The rate of sequence evolution of the minicircle component of the kDNA is extremely rapid and the function of the minicircle is as yet unknown [17–19]; different strains of the same species have been shown to differ considerably in minicircle sequences [20]. Maxicircle DNA sequences are more conserved, but small variations have been reported for different *brucei* strains [10, 14, 15]; maxicircle DNA appears to represent the homologue of the informational mtDNA molecules found in other eukaryotes [17].

The unusual nature of the kDNA of the trypanosomes may have some relation to the unique biphasic parasitic life cycle of these protozoa, which involves the alternating loss and biogenesis of mitochondrial enzymes in the different stages [1]. This hypothesis is consistent with the finding of Riou and Saucier [16] of an apparent absence of kDNA minicircle sequence heterogeneity in a strain of *T. equiperdum*, a species that lacks an insect vector phase of the life cycle.

Some of these results have been reported in abstract form (6th Annual Meeting on Basic Research on Chagas Disease, Caxambu, Brazil, 1979) and in a symposium summary paper [21].

MATERIALS AND METHODS

Cells and cell culture. Clonal strains of *T. brucei*, STIB 367H and STIB 366D were kindly provided by Dr. Leo Jenni of the Swiss Tropical Institute in Basel in September, 1976. STIB 367H is a clone from Lump 227. STIB 366D is a clone from S42/030 which was isolated in 1966 from a warthog in Uganda. Both STIB 367H and 366D were derived from single metacyclic trypanosomes from infected tsetse flies injected into irradiated mice. In our laboratory at UCLA, bloodstream trypanosomes were obtained by infecting γ -irradiated rats (800 rad) with 1×10^6 trypanosomes from frozen stabilates and harvesting the blood at peak parasitemia. The trypanosomes were isolated by the DEAE-cellulose method [22].

Bloodstream trypanosomes of strain 366D were cultured in the presence of a γ -irradiated (5000 rad) fetal lung fibroblast cell line (Flow F2000) in RPMI 1640 medium supplemented with 10% fetal calf serum by the method of Hirumi et al. [23].

Procyclic trypanosome cultures of strains 366D and 367H were obtained by transformation of bloodstream forms from the rat in modified Steiger's medium at cell densities of $3-5 \times 10^6$ /ml as described previously [24]. The procyclic cultures were grown at 27°C in rolling bottles or T25 flasks. Maximum cell densities of $50-70 \times 10^6$ cells/ml were obtained.

Isolation of kDNA. Cells were harvested and washed in cold 0.15 M NaCl–0.1 M EDTA. kDNA was isolated by the hot sarkosyl-pronase method [8, 25] with the addition of two successive EthBr–CsCl gradients. Mild shearing of the lysate was performed to fragment nuclear DNA. The yield of closed network kDNA from procyclic trypanosomes or bloodstream forms varied from 1.2 to 4.0 μ g/ 10^9 cells. Unless otherwise indicated, all results shown in figures refer to kDNA from strain 366D procyclic cells.

Isolation of maxicircle DNA. This was performed by Hoechst 33258 dye–CsCl centrifugation of Bam HI or Pst I digested network DNA as described previously [25]. A no. 50 Beckman anglehead rotor was used to achieve a greater separation of the bands. Two successive gradients were used to achieve complete purification (Fig. 2).

Sonication of network DNA. This was performed as described previously [8]. *T. brucei* kDNA from procyclic cells was labeled in vivo with 10 μ Ci/ml [3 H]thymidine. The kDNA was isolated, sonicated briefly and cosedimented in 5–20% alkaline sucrose gradients with sonicated unlabeled *Leishmania tarentolae* kDNA as an internal A_{260} marker. The *L. tarentolae* kDNA was isolated as described previously [8].

Restriction enzyme digestions and gel electrophoresis. The digestions were performed with excess enzyme in the buffers suggested by the supplier, New England Biolabs. After digestion the fragments were ethanol-precipitated and resuspended in loading buffer for electrophoresis as described previously [9, 26]. Electrophoresis was performed in horizontal agarose gels or in vertical acrylamide gradient gels as described previously [9]. Photography of gels was as described previously [27]. Reference molecular weights are as follows: λ DNA – 49 kb; T₇ DNA – 38 kb; λ /Hind III – 23.7, 9.5, 6.6, 4.3, 2.1, 1.9 kb; ϕ XRF/Hae III – 1353, 1078, 872, 603, 310, 271, 281, 234, 194, 118, 72 base pairs (Bethesda Research Labs, 1980 Catalog).

Isolation of kinetoplast-mitochondrion fraction. This was performed as described previously [17, 28]. The kinetoplast-mitochondrial fraction from procyclic cells banded in approximately the same location in the Renografin gradient as that from *L. tarentolae*.

Isolation of RNA. This was performed as described previously [17]. Methylmercury hydroxide agarose denaturing gels were run and RNAs were eluted from gels and labeled in vitro with 32 P as described previously [17].

Southern transfers and hybridizations. Two methods were used for transfer of DNA fragments from agarose to filters and hybridization with ^{32}P -labeled probes. Method 1: After staining with EthBr and photography, the gel was irradiated with short wavelength UV (Model C51 without filter at distance of 14 cm, UV Products, Inc., San Gabriel, CA) for 10 min, denatured in 0.5 NaOH for 45 min and neutralized in 0.14 M citrate-phosphate buffer (pH 4) for 1 h. The gel was then blotted onto diazotized [29] APT paper (aminophenylthioether paper, prepared by a method provided by Brian Seed, pers. commun.) with 0.02 M citrate-phosphate buffer (pH 4). The filter was preincubated in 50% formamide, 5 \times SSC (0.75 M NaCl/0.075 M sodium citrate, pH 7.0), 0.2% sodium-dodecyl sulfonate, 0.02% bovine serum albumin, 0.02% Ficoll and 0.02% polyvinylpyrrolidone [30], 1% glycine, at 42°C for 4 h. Hybridization was then performed in 2 \times SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) at 47°C with ^{32}P -labeled RNA probes for 24 h. The filters were then washed in 2 \times SSC at 25°C and incubated with RNAase A + T1 (20 $\mu\text{g}/\text{ml}$ and 20 u/ml) for 30 min at 25°C, and then washed again in 2 \times SSC. Exposure to preflashed Kodak RP-5 film in the presence of a Cronex Quanta II intensifying screen was for several days at -70°C. Method 2: The gel was blotted as above, but the hybridization was performed in prehybridization buffer minus glycine at 37°C for 56 h. The filters were washed in 50% formamide, 2 \times SSC at 25°C for 2 h and then in 2 \times SSC for 1 h.

Cloning and colony hybridization. kDNA from 366D procyclic cells was digested with Bam H1 or Hind III, and the fragments were ligated to Bam H1 or Hind III-linearized pBR322 plasmid DNA. Ligation, transfection of *Escherichia coli* RR1 cells and double antibiotic selection of recombinant clones were performed as described previously [26]. Amp^r Tet^s clones were then selected for the presence of kDNA sequences by colony hybridization, using ^{32}P -labeled nick translated total kDNA or nick translated gel purified Bam H1 maxicircle DNA as probes. All Bam H1 recombinant clones proved to contain minicircle inserts. Several Hind III clones contained maxicircle inserts, which were sized by gel electrophoresis of digested purified plasmid DNA. Colony hybridization and nick translation of DNA probes were performed as described previously [26]. The specific activity of the DNA probes was approx. 5×10^7 cpm/ μg .

RESULTS

Network kDNA-isolation

Closed network kDNA was isolated by our standard hot sarkosyl-pronase method, including a final EthBr-CsCl banding step. In vivo [^3H]thymidine labeled network DNA was subjected to band sedimentation in neutral sucrose gradients together with *L. tarentolae* network DNA as an *s* value marker. The *T. brucei* network DNA sedimented with a major peak of approx. 1700 S and a minor peak of approx. 2200 S implying a high degree of integrity of the isolated network DNA.

Maxicircle DNA-isolation

Total cell DNA in buoyant CsCl showed a major peak of nuclear DNA at 1.708 g/ml, a nuclear satellite peak at 1.702 g/ml and a high molecular weight kDNA network peak at 1.690 g/ml, as reported previously [10]. Digestion of purified covalently closed kDNA with Pst I or Bam HI released a minor band with a density of 1.682 g/ml representing approx. 39% of the network DNA (Fig. 1). A minor low density band (1.664–1.669 g/ml) was reproducibly released (also by Hpa II) but this was not studied further.

The 1.682 g/ml band consisted of linearized unit length maxicircle DNA as shown by agarose gel electrophoresis. This linearized maxicircle DNA could be isolated by the Hoechst dye–CsCl method [25] (Fig. 2). Comigration of the Pst I or Bam HI linearized

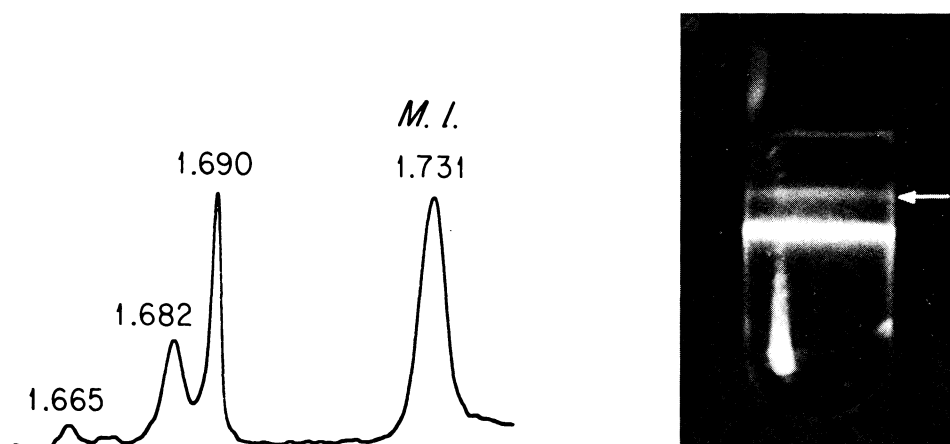


Fig. 1. Analytical CsCl buoyant analysis of 366D procyclic *T. brucei* kDNA digested with Pst I. The reference DNA is from *Micrococcus lysodeikticus* (M.l.)

Fig. 2. Hoechst 33258–CsCl gradient of Bam HI digested 366D procyclic *T. brucei* kDNA. Conditions: no. 50 rotor, 40 h, 40 000 rpm, 20°C, long wavelength UV illumination, initial $N_D^{25^\circ} = 1.3935$. The maxicircle band is indicated by the arrow.

T. brucei maxicircle DNA with Eco RI linearized *L. tarentolae* maxicircle DNA in 0.5% agarose showed the *T. brucei* maxicircle DNA to be smaller than that of *L. tarentolae* (Fig. 3). In this same gel the Eco RI, Hae III and Hind III digests of *T. brucei* circular maxicircle DNA are also presented.

Minicircle DNA-isolation

Closed monomeric minicircles were isolated by alkaline band sedimentation of sonicated closed network DNA. Cosedimentation of closed monomeric minicircles with closed minicircles of *L. tarentolae* kDNA (870 base pairs [7]) yielded a molecular size of approx. 1100 base pairs.

Minicircle DNA was also released from network DNA by digestion with several restriction enzymes: Hind III, Hha I, Eco RI, Bgl II, Hae III, Pst I, Hpa II and Bam HI. The relatively extreme sequence heterogeneity of minicircle DNA in *T. brucei* noted by others [10, 12, 15, 20] was evidenced by the limited release of unit length linearized minicircles by any of the enzymes tested. Maxicircle fragments were also evident as were undigested catenanes remaining at the origin (Fig. 4). The apparently single minicircle bands seen in agarose gels were shown by acrylamide gradient gel electrophoresis to consist of 5–10 closely spaced bands, implying a substantial minicircle minor length hetero-

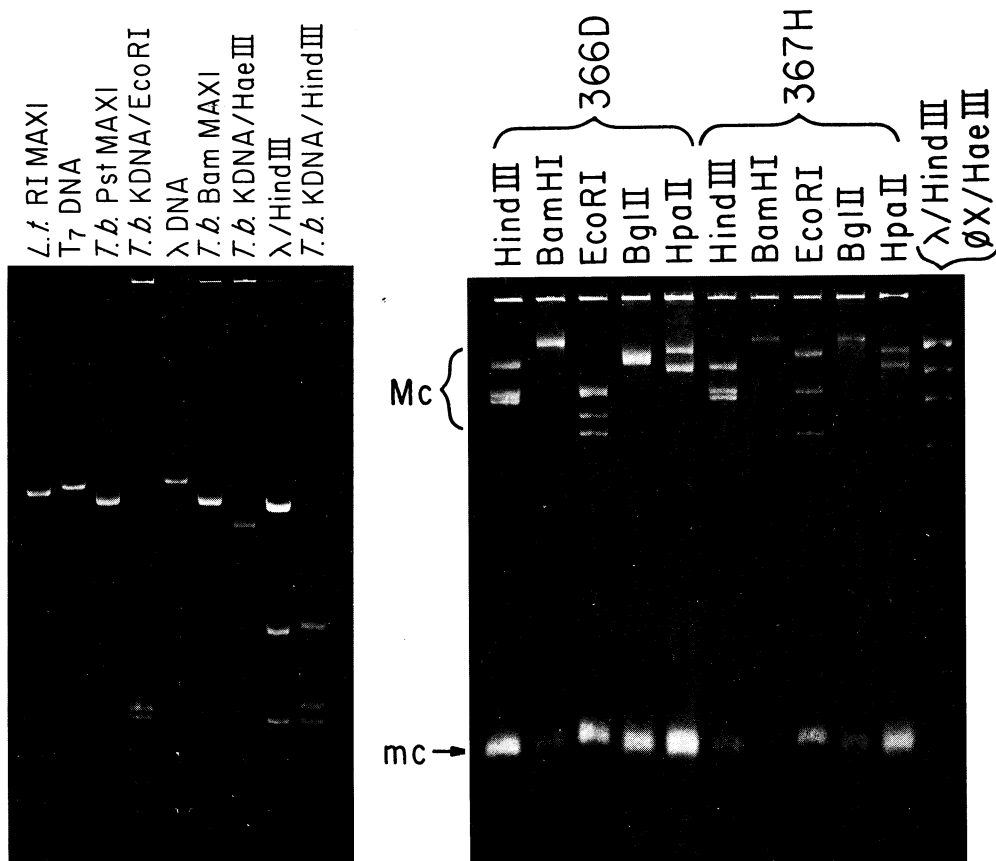


Fig. 3. Comparison of mobility of unit length 366D procyclic *T. brucei* (T.b.) maxicircle DNA with Eco RI linearized unit length *L. tarentolae* (L.t.) maxicircle DNA (30 kb [26]) in 0.5% agarose. *T. brucei* kDNA digested with Hind III, Eco RI and Hae III is also included. The molecular weight references are *T*₇ DNA, λDNA and λ/Hind III fragments.

Fig. 4. Comparison of digestions of kDNA from two clonal strains (366D and 367H) of *T. brucei* procyclics in 0.8% agarose. The minicircle (mc) and maxicircle (Mc) regions are indicated by arrows. The molecular weight references are λ/Hind III and φXRF/Hae III fragments.

geneity more extensive than that previously found in *Phytomonas* [9], *Crithidia* [5] or *Leishmania* [25] (Fig. 5).

Bam H1-linearized minicircles were cloned into pBR322, and 25 Amp^r Tet^s minicircle clones were selected by colony hybridization with nick translated total kDNA probe and by direct gel analysis of insert size after Bam H1 digestion (data not shown). The minicircle inserts in clones pTb-5 and pTb-7 were isolated by isopycnic banding in Hoechst 33258–CsCl gradients [25] and the insert pTb-7 was labeled in vitro with ³²P and used as a hybridization probe in colony hybridization. Four of the 25 minicircle clones showed substantial sequence homology with clone pTb-7, but all showed some extent of homology. This indicates the existence of more than one semihomologous minicircle sequence class possessing a single Bam H1 site. No hybridization was observed with a ³²P-labeled total minicircle probe from *L. tarentolae* kDNA implying a lack of cross species minicircle sequence homology at least for those particular clones (Fig. 7).

Restriction mapping and cloning of maxicircle DNA

Restriction enzyme profiles of kDNA from bloodstream forms or procyclic culture forms of a single strain were identical (data not shown). However, differences were

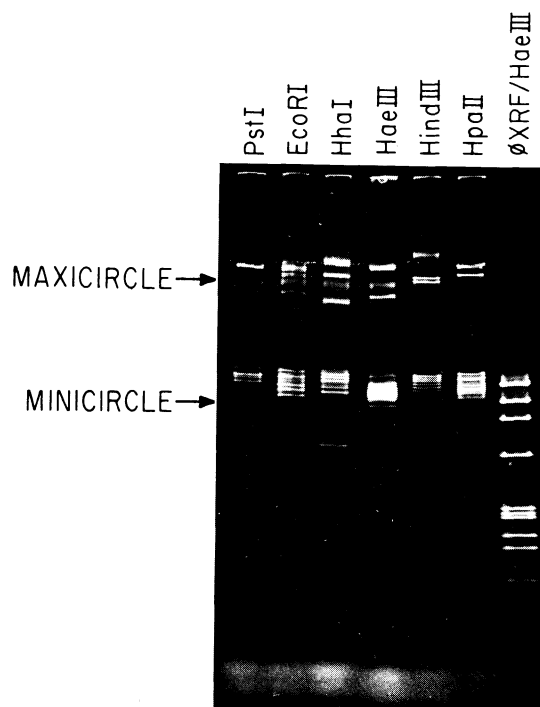


Fig. 5. Demonstration of heterogeneity of the major unit length minicircle band 366D procyclic *T. brucei* kDNA in an acrylamide gradient gel. Conditions: 3.5–10% acrylamide with a 3.0% stacking region. The molecular weight references are ϕ XRF/Hae III fragments.

apparent in the maxicircle region of the kDNA restriction profiles of the two clonal strains examined, 367H and 366D (Fig. 4). The differences could be explained by the deletion of a single Eco RI site and a single Bgl II site in 367H maxicircle DNA. Minicircle patterns were uninformative due to the limited extent of digestion.

A restriction map of the maxicircle of 366D was constructed for several enzymes by means of both double and triple digestions and the RNA Southern hybridizations described below (Fig. 6). Digestions were performed both on total network DNA and on purified linearized maxicircle DNA. The restriction map was constructed by using an assumed total size of 28 kb which was obtained by averaging the summations of fragments produced by the following enzymes: Hpa II + Bam HI, Hpa II + Pst I, Bgl II + Bam HI, Bgl II + Pst I, Pst I + Bam HI, Hind III + Bam HI, Eco RI + Bam HI, Eco RI + Pst I, Hha I + Bam HI, Hha I + Pst I. However, comigration of digested maxicircle DNA from strain 164 *T. brucei* (kindly provided by Dr. Kenneth Stuart) with digested maxicircle DNA from strain 366D showed that the only difference in fragment size was in the third Eco RI fragment. The third Eco RI fragment of strain 366D was 200–300 base pairs larger than that of strain 164. Furthermore, following a suggestion of Dr. Piet Borst (pers. commun.) size normalization of the published maxicircle restriction maps of *T. brucei* strains 164 and 427-60 to 20 kb yielded an excellent fit of most restriction sites. Therefore we concluded that our original 28 kb size was an overestimate due to size calibration error or to anomalous migration of certain maxicircle fragments in our gel system. Size normalization of our 366D maxicircle map to the 427-60 map yielded an excellent fit of most restriction sites.

The two smaller Hind III maxicircle fragments, B and C, of 366D were cloned into the bacterial plasmid pBR322. Colony hybridization of the cloned maxicircle fragments indicated sequence homology of the cloned Hind III C fragment with purified *L. tarentolae* maxicircle DNA probe and especially with the cloned maxicircle fragment of *L. tarentolae* kDNA (pLt-120) that contains the 9 and 12 S kRNA genes [24] (Fig. 7). Digestions of the cloned Hind III maxicircle fragments aided in the construction of the restriction map in Fig. 6. The lack of visible hybridization of the pLt-120 and pLt-30 clones with the total kDNA probe in Fig. 7A is due to the low percentage (approx. 5%) of maxicircle DNA in total kDNA of *L. tarentolae* [25].

Isolation of kRNA

A kinetoplast-mitochondrion fraction was isolated from procyclic culture forms of strain 367H (and 366D) by the Renografin flotation method. Total kRNA was isolated from this fraction as described in Materials and Methods. Two major RNA species were seen in nondenaturing 5–10% acrylamide gels, the smaller of which migrated somewhat faster than the 9 S kRNA of *L. tarentolae* and the larger of which migrated somewhat slower than the 12 S kRNA of *L. tarentolae* (Fig. 8). These differences were due to secondary structure since in methylmercury hydroxide-agarose denaturing gels, the *T. brucei* kRNAs comigrated with the *L. tarentolae* kRNAs (Fig. 9).

Two RNAs with the same mobility as the 9 and 12 S kRNAs were observed in 5%

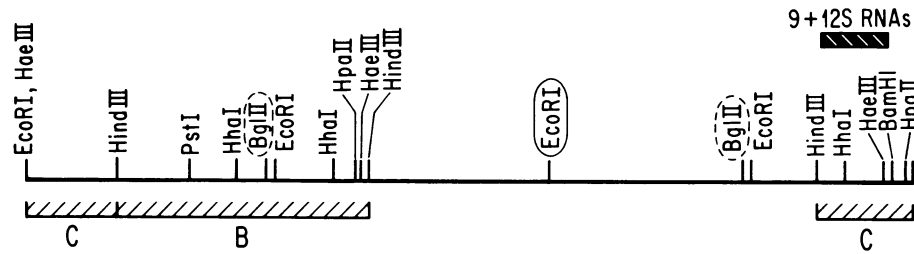


Fig. 6. Restriction map of the kDNA maxicircle from strain 366D. The molecule was arbitrarily linearized at an Eco RI site. The positions of the 9 and 12 S genes are indicated, as are the B and C Hind III fragments that have been cloned in pBR322. The circled Eco RI site and one of the circled Bgl II sites is missing in the 367H maxicircle DNA. Total length set at 20 kb.

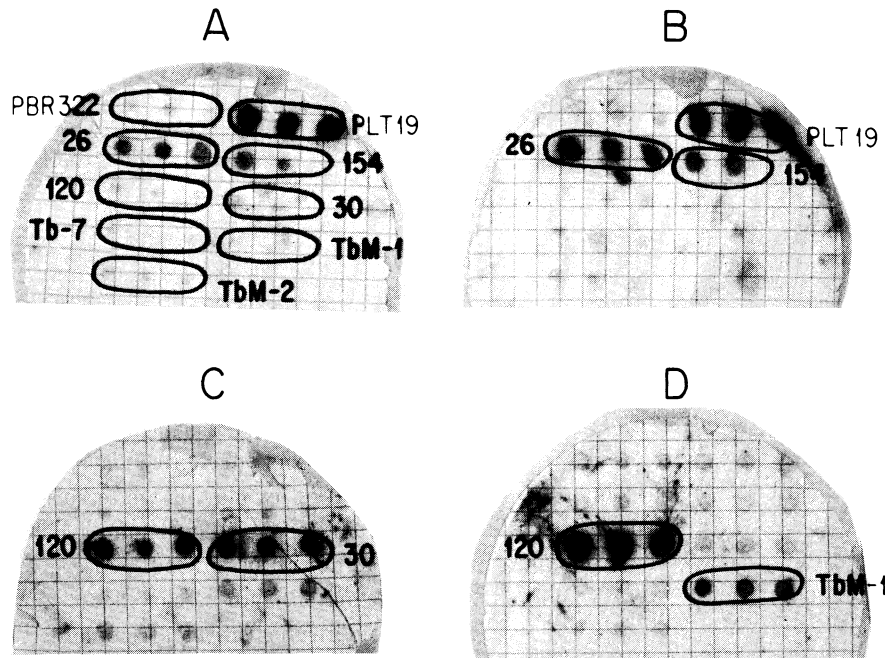


Fig. 7. Colony hybridization of recombinant clones containing *L. tarentolae* kDNA fragments and 366D procyclic *T. brucei* kDNA fragments. The clones were spotted in triplicate on each of four filters as indicated. The filters were processed as described in Materials and Methods. The ^{32}P -labeled probes were: A. Total kDNA from *L. tarentolae*. B. Total closed monomeric minicircle DNA from *L. tarentolae*. C. Purified Bam HI-linearized maxicircle DNA from *L. tarentolae*. D. Purified insert DNA from pLt-120 plasmid which contains a 4.4×10^6 dalton fragment of *L. tarentolae* maxicircle DNA which contains the 9 and 12 S RNA genes [24]. pLt-19, 26 and 154 are *L. tarentolae* kDNA unit length minicircle clones; pLt-120 and 30 are *L. tarentolae* Eco RI + Bam HI and Hind III maxicircle clones. TbM-1 is the Hind III C fragment maxicircle clone from *T. brucei*; TbM-2 is the Hind III B fragment maxicircle clone from *T. brucei*. Tb-7 is a unit length minicircle clone from *T. brucei*. The original filters were inserted under the autoradiographs for photography. The relative positions of the colonies are as indicated in A by circling the three colonies of each type. In B, C and D only those colonies showing strong positives are circled.

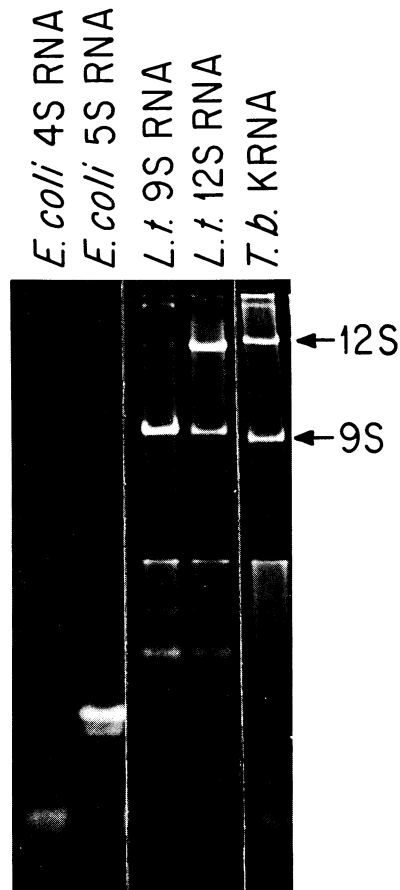


Fig. 8. Nondenaturing acrylamide gel of 367H *T. brucei* (T.b.) kRNA. *L. tarentolae* (L.t.) partially purified 9 and 12 S kRNAs and *E. coli* 4 S and 5 S RNA were corun as standards. Conditions: upper layer 5% acrylamide, lower layer 10% acrylamide.

acrylamide gels of total cell RNA from cultured bloodstream trypanosomes of strain 366D (data not shown).

Purified 9 and 12 S kRNAs of procyclic *T. brucei* were obtained by elution of ethidium-stained bands from a 5% acrylamide gel and were labeled in vitro after mild alkali hydrolysis using 5'-kinase with ^{32}P . These labeled RNAs were used as hybridization probes in Southern transfer experiments to localize the transcriptional origins on the kDNA maxicircle. Agarose gel profiles of total kDNA digested with Hind III, Hae III or Hha I were blotted onto filters and the filters hybridized with *T. brucei* 9 S RNA, *T. brucei* 12 S RNA, and *L. tarentolae* 9 + 12 S RNA labeled probes (Fig. 10). Specific maxicircle hybridization of all probes was observed. The heterologous *L. tarentolae* kRNAs hybridized to the same restriction fragments as the homologous *brucei* probes.

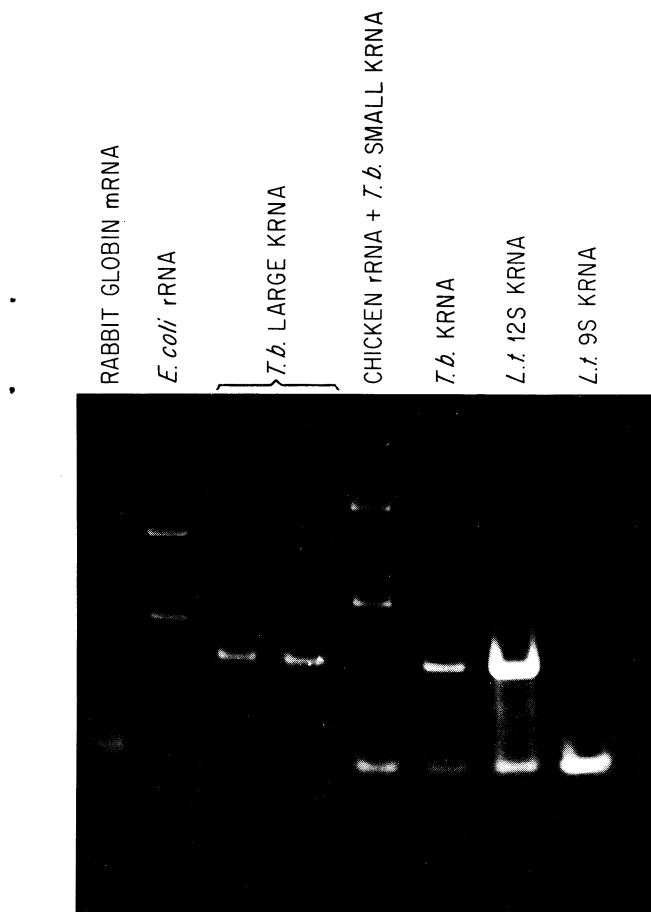


Fig. 9. Methylmercury hydroxide agarose gel (1.5%) of *T. brucei* kRNA. The size standards were *E. coli* rRNA, chicken rRNA, rabbit globin mRNA, and *L. tarentolae* 9 and 12 S kRNA. Note that the *L. tarentolae* '12 S' RNA is contaminated with 9 S RNA, and that the *T. brucei* small kRNA was corun with the chicken rRNA. The *T. brucei* large and small kRNAs were purified by elution of ethidium bromide stained 5% acrylamide gels.

A similar experiment was performed with the cloned maxicircle Hind III fragments (Fig. 11). Purified cloned Hind III fragment C was digested with Hpa II, Hae III and Bam H1 and run in agarose. Purified cloned fragment B was also run as a control. The gel was blotted onto a filter and the filter hybridized with 32 P-labeled *T. brucei* 9 and 12 S kRNA probes. Hybridization was observed only with fragment C and with subfragments of this fragment. Localization of the 9 and 12 S probes was identical. These blotting results allowed the approximate localizations of the 9 and 12 S genes on the restriction map as shown in Fig. 6.

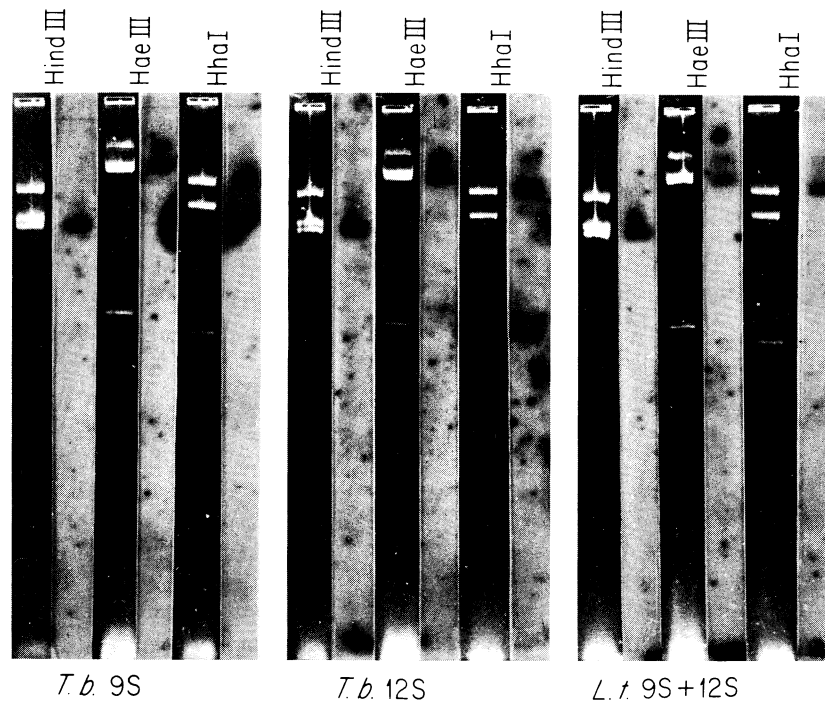


Fig. 10. Hybridization of in vitro labeled 367H *T. brucei* large and small kRNAs and *L. tarentolae* 9 and 12 S kRNAs to Southern blots of total kDNA digested with several enzymes. The gel was 1% agarose.

DISCUSSION

Our preliminary characterization of the kDNA of *T. brucei* was carried out as the initiation of a project to study the possibility of transcriptional regulation of kDNA genetic activity during the biphasic life cycle of this organism. During the course of this work, several studies [10, 14, 15, 31] on the kDNA of *T. brucei* appeared. Our results confirm and extend the results of these authors and also provide several new observations. The high AT content of maxicircle DNA relative to the minicircle DNA has proven to be a general phenomenon among the hemoflagellates [14–16, 25]. In *T. brucei* the difference is less than in the lower hemoflagellates but is still sufficient to allow the isolation of the linearized maxicircle DNA by the Hoechst dye–CsCl method [25]. In a comparison of the kDNA in the two stages of the life cycle in a single strain (bloodstream forms and procyclic culture forms), no differences in maxicircle restriction sites were apparent, and a detailed restriction map of the maxicircle DNA of the procyclic form of strain 366D was derived. However, minor differences were observed in the number of restriction sites on the maxicircle DNA of strains 366D and 367H, and these dif-

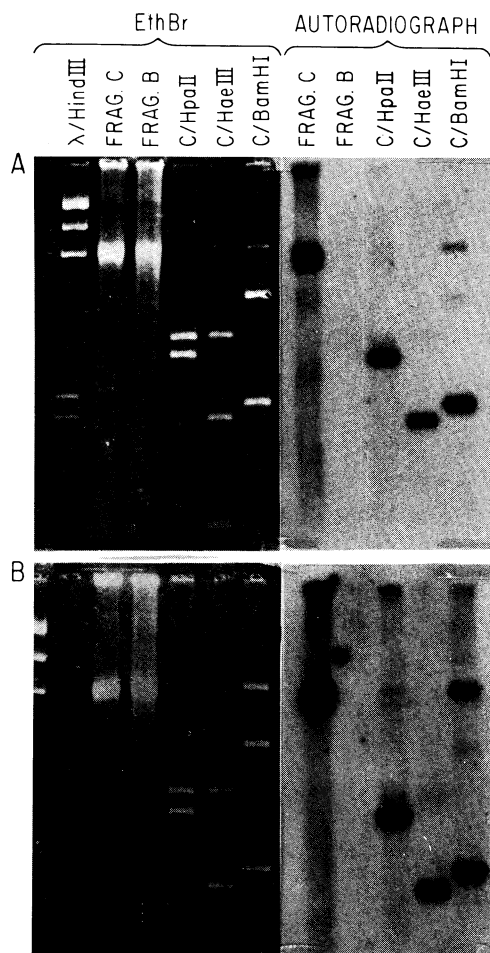


Fig. 11. Hybridization of in vitro labeled 376H *T. brucei* large and small kRNAs to Southern blots of cloned Hind III maxicircle fragments B and C digested with several enzymes. A, small RNA probe. B, large RNA probe.

ferences could be explained by the loss of single sites for Eco RI and Bgl II in the DNA of 367H as indicated in Fig. 6.

As mentioned in the Results section, when the three maxicircle maps for *T. brucei* strains 366D, 164 and 427-60 are normalized to the same size (20 kb), there is an excellent fit of most restriction sites. The 0.2–0.3 kb size variation between 366D and 164 occurs within the so-called 'silent region' [14] of the map that has few restriction sites. By analogy with the AT heterogeneity in the maxicircle of *L. tarentolae*, this region may prove to contain a higher percentage of AT than the remainder of the molecule.

Two Hind III fragments of the maxicircle DNA of 366D were cloned in the bacterial plasmid, pBR322, including the fragment that contains the 9 and 12 S RNA genes.

Several semihomologous Bam H1 cut minicircles were also cloned in pBR322. No homology was observed between the cloned *brucei* minicircles and total minicircle DNA from *L. tarentolae*.

kRNAs have been isolated that comigrate in denaturing gels with the 9 and 12 S kRNAs from *L. tarentolae*. The genes have been localized on the maxicircle restriction map of 366D and were shown to cross hybridize with the 9 and 12 S maxicircle genes of *L. tarentolae*. The genes map near to each other as is the case in *L. tarentolae*. Although no evidence was presented in this paper that the 9 and 12 S genes are distinct and not overlapping, evidence has been obtained for the distinct nature of the homologous genes in the maxicircle DNA of *L. tarentolae* [33] and there is no reason to believe that this differs in *T. brucei*. Borst and Hoeijmakers [32] have reported a predominant hybridization of in vivo labeled total cell *T. brucei* RNA with a 2000 nucleotide *T. brucei* maxicircle fragment. They also showed that this sequence is conserved in trypanosome evolution since *T. brucei* RNA cross hybridizes with the predominantly transcribed segment of *C. fasciculata* maxicircle DNA [18]. This segment corresponds to the 9 and 12 S maxicircle genes that we have localized. The presence of conserved 9 and 12 S RNAs in the kinetoplast seems to be universal among the hemoflagellates; we have isolated these RNAs from *L. tarentolae* [17], *P. davidi* [9], *C. fasciculata* (unpubl. exper.) and now *T. brucei*. The evolutionary sequence conservation of these genes, the absence of polyadenylation of the RNA transcripts, and the fact that the 9 and 12 S transcripts are the major stable RNA species in the mitochondrial fraction all imply a mitochondrial ribosomal role, but this conclusion has to be verified by the isolation and characterization of kinetoplast ribosomes, which has proven to be a difficult problem.

The presence of 9 and 12 S kRNAs was demonstrated in cultured bloodstream trypanosomes which lack a mitochondrial respiratory chain as well as in procyclic culture forms which contain a full complement of mitochondrial respiratory enzymes. This result is consistent with the hybridization results of Borst and Hoeijmakers [32] since they used RNA from bloodstream trypanosomes for their maxicircle hybridization studies.

ACKNOWLEDGEMENTS

We wish to acknowledge the assistance of R. LePage in the establishment of the cultures of bloodstream trypanosomes and for suggesting the use of irradiated feeder cells. We also thank June Baumer for assistance with analytical centrifugation, Brian Seed for a preprint of his APT paper procedure, Leo Jenni for supplying the *T. brucei* clonal strains, George Hill for discussion, Ken Stuart for exchanging kDNA of 164, and Piet Borst for unpublished results. This research was supported by research grants from the National Institute of Allergies and Infectious Diseases (AI-09102, AI-13027).

REFERENCES

- [1] Simpson, L. (1972) The kinetoplast of the hemoflagellates. *Int. Rev. Cytol.* 32, 130–207.
- [2] Borst, P. and Hoeijmakers, J. (1979) Kinetoplast DNA. *Plasmid* 2, 20–40.
- [3] Renger, H. and Wolstenholme, D. (1972) The form and structure of kinetoplast DNA of *Crithidia*. *J. Cell Biol.* 54, 346–364.
- [4] Simpson, A.M. and Simpson, L. (1974) Isolation and characterization of kinetoplast DNA networks and minicircle from *Crithidia fasciculata*. *J. Protozool.* 21, 774–781.
- [5] Kleisen, C., Borst, P. and Weijers, P. (1976) The minicircles of *Crithidia luciliae* are heterogeneous in base sequence. *Eur. J. Biochem.* 64, 141–151.
- [6] Weislogel, P., Hoeijmakers, J., Fairlamb, A., Kleisen, C. and Borst, P. (1977) Characterization of kinetoplast DNA networks from the insect trypanosome *Crithidia luciliae*. *Biochim. Biophys. Acta* 478, 167–179.
- [7] Simpson, L. and da Silva A. (1971) Isolation and characterization of kinetoplast DNA from *Leishmania tarentolae*. *J. Mol. Biol.* 56, 443–473.
- [8] Simpson, L. and Berliner, J. (1974) Isolation of the kinetoplast DNA of *Leishmania tarentolae* in the form of a network. *J. Protozool.* 21, 382–393.
- [9] Cheng, D. and Simpson, L. (1978) Isolation and characterization of kinetoplast DNA and RNA of *Phytomonas davidi*. *Plasmid* 1, 197–315.
- [10] Fairlamb, A., Weislogel, P., Hoeijmakers, J. and Borst, P. (1978) Isolation and characterization of kinetoplast DNA from bloodstream form of *Trypanosoma brucei*. *J. Cell Biol.* 76, 293–309.
- [11] Leon, W., Frank, A., Hoeijmakers, J., Fase-Fowler, F., Borst, P., Brunel, F. and Davison, J. (1980) Maxi-circles and mini-circles in kinetoplast DNA from *Trypanosoma cruzi*. *Biochim. Biophys. Acta* 607, 221–231.
- [12] Donelson, J., Majiwa, F. and Williams, R. (1979) Kinetoplast DNA minicircles of *Trypanosoma brucei* share regions of sequence homology. *Plasmid* 2, 572–588.
- [13] Chen, K. and Donelson, J. (1980) The sequences of two kinetoplast DNA minicircles of *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. U.S.A.*, in press.
- [14] Borst, P. and Fase-Fowler, F. (1979) The maxicircle of *Trypanosoma brucei* kinetoplast DNA. *Biochim. Biophys. Acta* 565, 1–12.
- [15] Stuart, K. (1979) Kinetoplast DNA of *Trypanosoma brucei*. Physical map of the maxicircle. *Plasmid* 2, 520–528.
- [16] Riou, G. and Saucier, J. (1979) Characterization of the molecular components in kinetoplast–mitochondrial DNA of *Trypanosoma equiperdum*. *J. Cell Biol.* 82, 248–263.
- [17] Simpson, L. and Simpson, A.M. (1978) Kinetoplast RNA of *Leishmania tarentolae*. *Cell* 14, 169–178.
- [18] Hoeijmakers, J.H.J. and Borst, P. (1978) RNA from the insect trypanosome *Crithidia luciliae* contains transcripts of the maxicircle and not of the minicircle component of kinetoplast DNA. *Biochim. Biophys. Acta* 521, 407–411.
- [19] Fouts, D. and Wolstenholme, D. (1975) Evidence for a partial RNA transcript of the small circular component kinetoplast DNA of *Crithidia acanthocephali*. *Nucl. Acids Res.* 6, 3785–3804.
- [20] Steinert, M., Van Assel, S., Borst, P. and Newton, B. (1976) Evolution of kinetoplast DNA. In: *The Genetic Function of Mitochondrial DNA*, (Saccone, C. and Kroon, A., eds.), pp. 71–81, Elsevier/North-Holland Biomedical Press, Amsterdam.
- [21] Simpson, L., Simpson, A.M., Masuda, H., Rosenblatt, H., Michael, N. and Kidane, G. (1979) Replication and transcription of kinetoplast DNA. In: *Extrachromosomal DNA. ICN–UCLA Symposium on Molecular and Cellular Biology 15*, (Borst, P., David, I., Weissman, S. and Fox, C.F., eds.), pp. 533–548, Academic Press, New York.
- [22] Lanham, S.M. (1968) Separation of trypanosomes from the blood of infected rats and mice by anion-exchangers. *Nature (London)* 218, 1273–1274.

- [23] Hirumi, H., Doyle, J. and Hirumi, K. (1977) African trypanosomes: Cultivation of animal-infective *Trypanosoma brucei* in vitro. *Science* 196, 992–994.
- [24] Simpson, L. (1978) Glucose-sensitive strain of *Trypanosoma brucei*. *J. Parasitol.* 64, 360.
- [25] Simpson, L. (1979) Isolation of maxicircle component of kinetoplast DNA from hemoflagellate protozoa. *Proc. Natl. Acad. Sci. U.S.A.* 76, 1585–1588.
- [26] Masuda, H., Simpson, L., Rosenblatt, H. and Simpson, A.M. (1979) Restriction map, partial cloning and localization of the 9S and 12S RNA genes on the maxicircle component of the kinetoplast DNA of *Leishmania tarentolae*. *Gene* 6, 51–73.
- [27] Brunk, C. and Simpson, L. (1977) Comparison of various UV sources for fluorescent detection of ethidium bromide–DNA complexes in polyacrylamide gels. *Anal. Biochem.* 82, 455–462.
- [28] Braly, P., Simpson, L. and Kretzer, F. (1974) Isolation of kinetoplast–mitochondrial complexes from *Leishmania tarentolae*. *J. Protozool.* 21, 782–790.
- [29] Alwine, J., Kemp, D. and Stark, G. (1977) Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5350–5354.
- [30] Denhardt, D. (1966) A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23, 641–646.
- [31] Brunel, F., Davidson, J., Merchez, M., Borst, P. and Weijers, P. (1979) The use of recombinant DNA techniques in the analysis of *Trypanosoma brucei* kinetoplast DNA. In: *FEBS symposium on DNA*, (Zadrazil, J. and Sparar, J., eds.), pp. 45–54, Pergamon Press, Oxford.
- [32] Borst, P. and Hoeijmakers, J. (1979) Structure and function of the kinetoplast DNA of the African trypanosomes. In: *Extrachromosomal DNA. ICN–UCLA Symposium on Molecular and Cellular Biology 15*, (Borst, P., David, I., Weissman, S. and Fox, C.F., eds.), pp. 515–531. Academic Press, New York.
- [33] Simpson, L., Simpson, A.M., Kidane, G., Livingston, L. and Spithill, T. (1980) The kinetoplast DNA of the hemoflagellate protozoa. *Am. J. Trop. Med. Hyg.*, in press.