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Chromosomes of Kinetoplastida

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We have compared chromosome-sized DNA molecules (molecular karyotypes) of five genera (nine species) of kinetoplastida after cell lysis and deproteinization of DNA in agarose blocks and size fractionation of the intact DNA molecules by pulsed field gradient (PFG) gel electrophoresis. With the possible exception of *Trypanosoma vivax* and *Crithidia fasciculata*, all species have at least 20 chromosomes. There are large differences between species in molecular karyotype and in the chromosomal distribution of the genes for α- and β-tubulin, rRNA and the common mini-exon sequence of kinetoplastid mRNAs. In all cases, the rRNA genes are in DNA that is larger than 500 kb. Whereas *T. brucei* has ~100 mini-chromosomes of 50–150 kb, only few are found in *T. equiperdum; T. vivax* has no DNA smaller than 2000 kb. As all three species exhibit antigenic variation, small chromosomes with telomeric variant surface glycoprotein genes cannot be vital to the mechanism of antigenic variation. The apparent plasticity of kinetoplastid genome composition makes PFG gel electrophoresis a potentially useful tool for taxonomic studies.

Key words: antigenic variation/karyotype/recombination/translocation/trypanosome

Introduction

African trypanosomes (genus *Trypanosoma*, see Figure 1) are unicellular parasites of vertebrates (Hoare, 1972; Lumsden and Evans, 1976). They survive in the bloodstream by periodically changing the antigenic composition of their cell surface coat which is made of a single type of protein, the variant surface glycoprotein or VSG (Cross, 1975, 1977, 1978; Capbern *et al.*, 1977; Vickerman, 1978). By the sequential expression of different VSG genes, the parasite varies its surface antigen (Hoeijmakers *et al.*, 1980; Borst and Cross, 1982; Borst *et al.*, 1983a, 1983b, 1983c).

Simple protists like *T. brucei* and other kinetoplastid flagellates have a 'primitive' mitotic apparatus. The chromosomes of these organisms do not fully condense in any phase of the mitotic cycle (Vickerman and Preston, 1970). Hence, microscopy cannot be used to obtain reliable information on the size and number of chromosomes, nor on the chromosomal location of genes.

A new approach to this problem is provided by a new technique, pulsed field gradient gel electrophoresis (PFG gel electrophoresis) developed by Schwartz and Cantor (Schwartz *et al.*, 1983; Schwartz and Cantor, 1984), which makes it possible to separate and analyse chromosome-sized molecules up to at least 2000 kb in agarose gels. We have used this technique to demonstrate that *T. brucei* has ~100 mini-chromosomes and several small chromosomes in between 200 kb and 2 mega-base pairs (Mb), (Van der Ploeg *et al.*, 1984a). Most, if not all of these contain telomeric VSG genes (Van der Ploeg *et al.*, 1984a). Recombinations, which involve transposition of hundreds of kilobase pairs, move telomeric VSG genes from chromosome to chromosome (Pays *et al.*, 1983; Van der Ploeg *et al.*, 1984b; Van der Ploeg and Cornelissen, 1984), resulting in striking differences in the size distribution of small chromosomes in different stocks of *T. brucei*. It is possible that these rearrangements play an essential role in the regulation of VSG gene expression (Van der Ploeg and Cornelissen, 1984).

These results have raised several questions. How representative is the molecular karyotype of *T. brucei* for kinetoplastid flagellates in general? Are mini-chromosomes essential for antigenic variation? Are molecular karyotypes useful for differentiating species in this order? To answer these questions we have size-fractionated DNA from nine species belonging to five genera of the family Trypanosomatidae (Figure 1) by PFG gel electrophoresis. We have characterized the resulting chromosome-sized DNA molecules by Southern blot analysis using cloned DNA probes for telomeres (Van der Ploeg *et al.*, 1984c; Blackburn and Challoner, 1984), the genes coding for the mini-exons of trypanosome mRNAs (De Lange *et al.*, 1983, 1984a, 1984b; Nelson *et al.*, 1983, 1984), tubulin genes (Thomashow *et al.*, 1983) and the rRNA genes of *T. brucei*.

Results

Trypanosomes exhibiting antigenic variation

Figure 2 shows an ethidium-stained agarose gel with chromosome-sized DNA molecules from three trypanosome species which exhibit antigenic variation. The diagonal migration of the DNA is due to the combination of electrical fields applied alternately in the North/South and West/East direction. The strangely bent lanes are due to the gradient field and the local effects of DNA concentration on the field. In this molecular karyogram the DNA from *T. brucei* (lane 1) separates into four main fractions, as previously reported (Van der Ploeg *et al.*, 1984a): large DNA that remains close to the slot; at least three chromosomes of 2 Mb; 5–7 chromosomes ranging in size between 200 kb and 2 Mb; and ~100 mini-chromosomes of 50–150 kb. There is an extensive zone of ethidium staining between the slot and the 2-Mb chromosomes. This consists partly of cellular debris and partly of chromosomes that tend to distribute between the slot and the 2-Mb chromosomes depending on the exact pulse frequencies and position of the DNA in the gel (cf. Van der Ploeg *et al.*,

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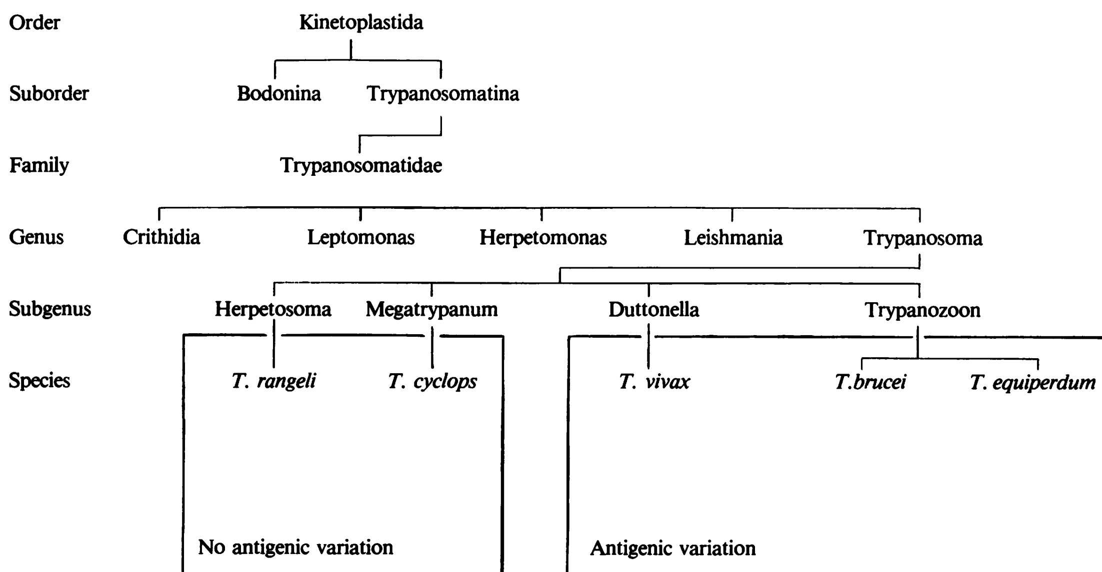

Fig. 1. Classification of species of the order of Kinetoplastida according to Hoare (1972). The figure only includes species and genera that have been tested in the chromosome separation analysis.

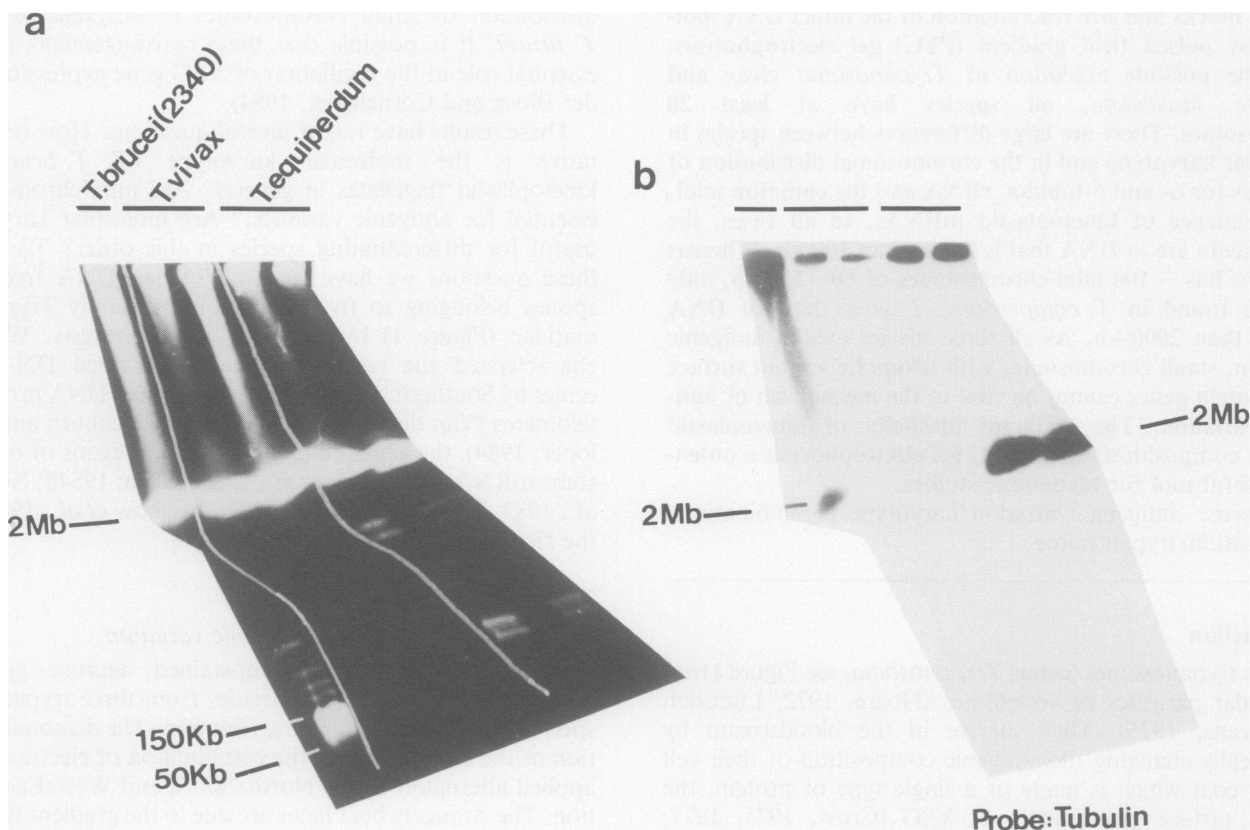

Fig. 2. PFG gel electrophoresis of trypanosomes exhibiting antigenic variation. **(a)** Trypanosomes were lysed and electrophoresed as described in Materials and methods at a pulse frequency of 35 s for 18 h. The gel was then stained with ethidium bromide. The gradient field and migration artefacts, due to the presence of different local DNA concentrations in the neighbouring lanes, are the cause of the abnormal curved path of the DNA through the gel. The correct comparison of the lanes is indicated with white lines. We have calibrated the mol. wts. of *T. brucei*, stock 427, DNA molecules by co-migration with yeast chromosomes and phage DNA (see Van der Ploeg *et al.*, 1984a) and used strain 427 DNA molecules as size markers in the experiments presented here. **(b)** The DNA was blotted on nitrocellulose filters and hybridized with the pTb $\alpha\beta$ T-1 tubulin probe (Thomashow *et al.*, 1983). Post-hybridizational washes were at 65°C and 0.1 x SSC.

1984a). The comparison of molecular karyotypes in Figure 2A shows rather striking differences in the complement of small chromosomes present in *T. brucei*, *T. vivax* and *T. equiperdum*. The abundance of mini-chromosomes is only found in *T. brucei*. *T. equiperdum* has ~10 small chromosomes (between 50 kb and 2 Mb) and *T. vivax* neither mini-chromosomes nor small chromosomes.

The distribution of several sequence elements over the try-

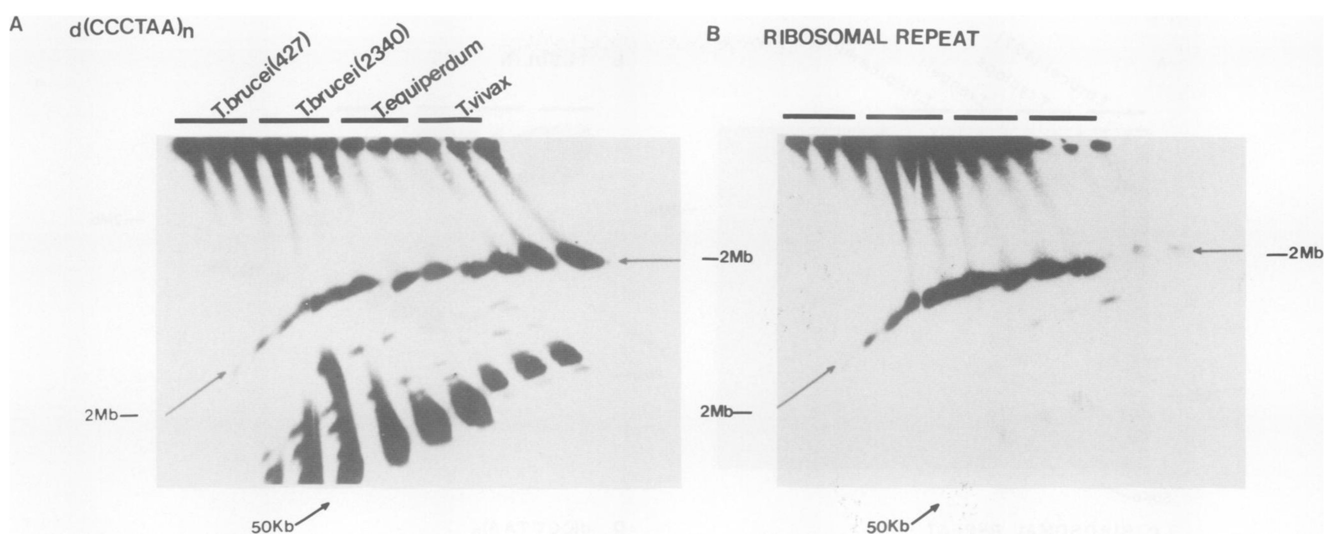

Fig. 3. PFG gel electrophoresis of trypanosomes exhibiting antigenic variation. Samples were lysed and electrophoresed as described in Materials and methods for 18 h at a pulse frequency of 35 s. The DNA was blotted onto nitrocellulose filters and hybridized with the (CCCTAA)n telomere-specific probe (Van der Ploeg *et al.*, 1984c; **panel A**) and the ribosomal repeat unit of *T. brucei* (**panel B**). Post-hybridizational washes were at 65°C and 3 x SSC.

panosome chromosomes is illustrated in Figures 2 and 3. Figure 3A shows that the chromosome-sized molecules of *T. equiperdum* and *T. vivax* give approximately stoichiometric hybridization with the telomere-specific (CCCTAA)n probe (Figure 3A), as previously shown for *T. brucei* (Van der Ploeg *et al.*, 1984a). *T. vivax* gives lower overall hybridization with this probe in blots of restriction-enzyme digested DNA than *T. brucei* (Van der Ploeg *et al.*, 1984c). Our present results show that this is attributable to the difference in chromosome number. Figure 2b shows the hybridization with a tubulin probe. Both *T. brucei* stocks (see also lanes 1–3 in Figure 4B) have the same hybridization pattern with the bulk of the material close to the slot and less in the 2-Mb chromosomes. In contrast, *T. equiperdum* has the hybridization both in the slot and the 2-Mb chromosomes, the latter hybridizing most intensely. In *T. vivax* the tubulin genes are only located in large DNA (Figure 2B). These results, which have been confirmed in other PFG gels, must reflect differences in the size of the chromosomes in which the tubulin genes are located. The results with the ribosomal DNA probe in Figure 3B are more uniform. With all three species the bulk of the hybridization is in large DNA tailing to the 2-Mb region. The detailed organization of rRNA genes in *T. brucei* has been established by Turner's group using Southern blotting and cloned DNA (Hasan *et al.*, 1982, 1984). There is also faint but reproducible hybridization in *T. equiperdum* and *T. brucei* on one intermediate-sized chromosome and on mini-chromosomes, respectively. We have not verified whether this hybridization is due to transcribed or spacer sequences.

Molecular karyotypes of trypanosomatids that do not exhibit antigenic variation

Figures 4 and 5 show the molecular karyotypes of other representatives of the genus *Trypanosoma* (*T. cyclops* and *T. rangeli*) and of other genera of the family Trypanosomatidae (*Leishmania, Leptomonas* and *Herpetomonas*; see Figure 1). Figure 5 illustrates that the exact separation of bands is critically dependent on the pulse time used, like in yeast (Schwartz and Cantor, 1984) and in *T. brucei* (Van der Ploeg *et al.*, 1984a). By comparing several PFG gels with different DNA concentrations and pulse frequencies, we deduced that the number of chromosomes exceeds 20 in all species analysed (see Table I). No chromosomes smaller than 700 kb were detected in any of these trypanosomatids. The striking differences in the size distribution of chromosome-sized DNA molecules illustrated by Figures 4A and 5A, are also reflected in the results obtained with gene-specific probes. In *T. rangeli* the tubulin genes are exclusively located on large DNA that remains close to the slot; in *T. cyclops* the tubulin genes are distributed between large DNA and a chromosome slightly smaller than 2 Mb; and in *Leishmania tropica minor* the tubulin genes are found in two locations in the gel (Figure 4B). The hybridization around the slot is probably an artefact. Figure 4C illustrates some of the extensive differences in the distribution of rRNA genes. All bands in the gel show roughly stoichiometric hybridization with the telomeric (CCCTAA)n probe (Figure 4D) indicating that each band represents intact linear chromosome-sized molecules. A summary of all results is presented in Table I. At a pulse frequency of 90 s all species analysed, except the African trypanosomes, yield DNA bands migrating slower than the 2-Mb band of *T. brucei* (see Figures 4 and 5). We have provisionally given extrapolated mol. wts. to these bands in Table I, but we have no direct evidence that migration behaviour in this part of the gel is a function of size. The data summarized in Table I have been reproduced at least three times without significant variation.

Discussion

Our analysis of several genera of the family of *Trypanosomatidae* by PFG gel electrophoresis has shown that chromosomes smaller than 700 kb only occur in the subgenus *Trypanozoon*. These chromosomes do not seem to be necessary for antigenic variation: *T. equiperdum* has few chromosomes in the mini-chromosome area and *T. vivax* has no small chromosomes at all, even though both species can vary their surface coat as does *T. brucei*. In fact, we have recently found that the number of small and mini-chromosomes in *T. brucei* itself also varies considerably between strains, three strains of *T. brucei gambiense* and one of

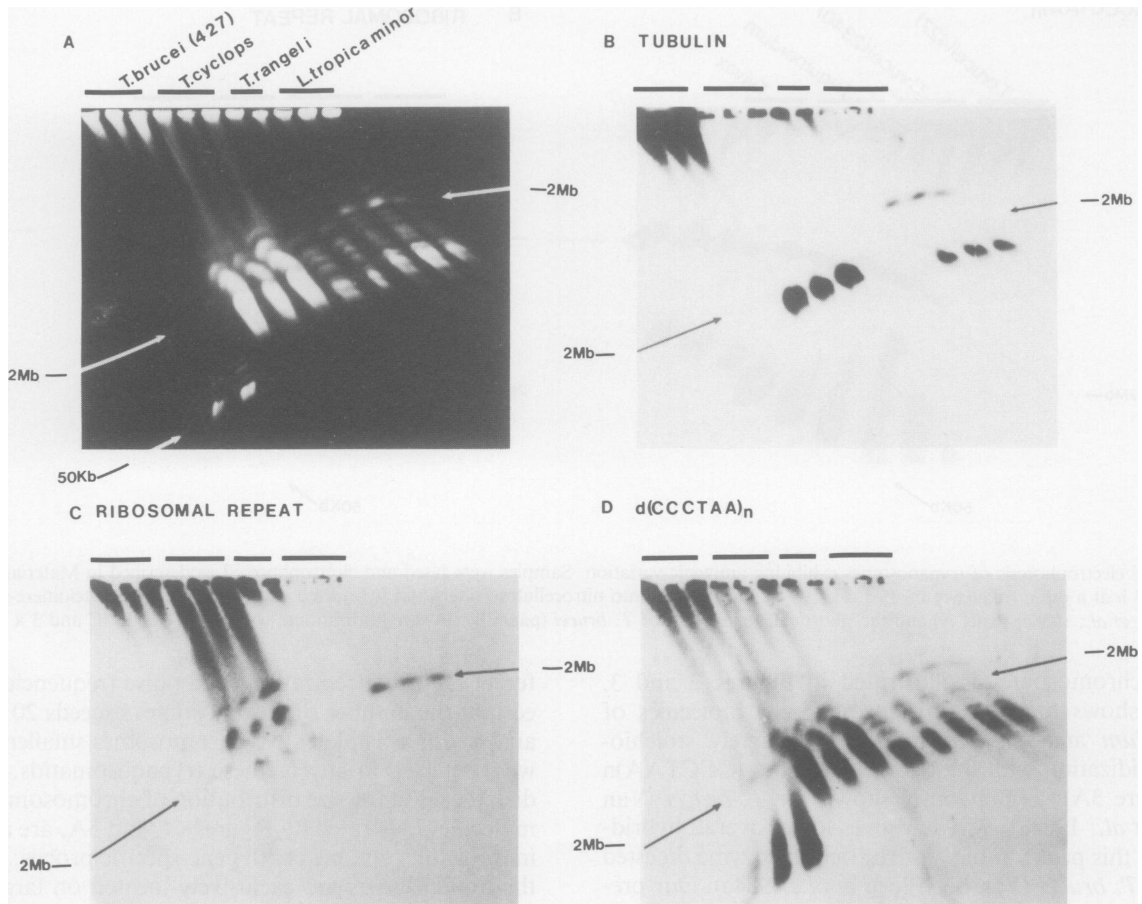

Fig. 4. Comparison of the molecular karyotypes of *T. brucei*, *T. cyclops*, *T. rangeli* and *L. tropica minor*. Samples were lysed and electrophoresed as described in Materials and methods for 18 h with a pulse frequency of 90 s. After electrophoresis, the gel was stained with ethidium bromide (**panel A**) and the DNA transferred to nitrocellulose filters and hybridized with the following probes: **panel B**, the pTb $\alpha\beta$ T-1 tubulin probe, post-hybridizational washes at 65°C and 0.1 x SSC, **panel C**, the ribosomal repeat unit of *T. brucei*; post-hybridizational washes at 65°C and 3 x SSC; **panel D**, the (CCCTAA)n telomere repeat of *T. brucei*; post-hybridizational washes at 65°C and 3 x SSC.

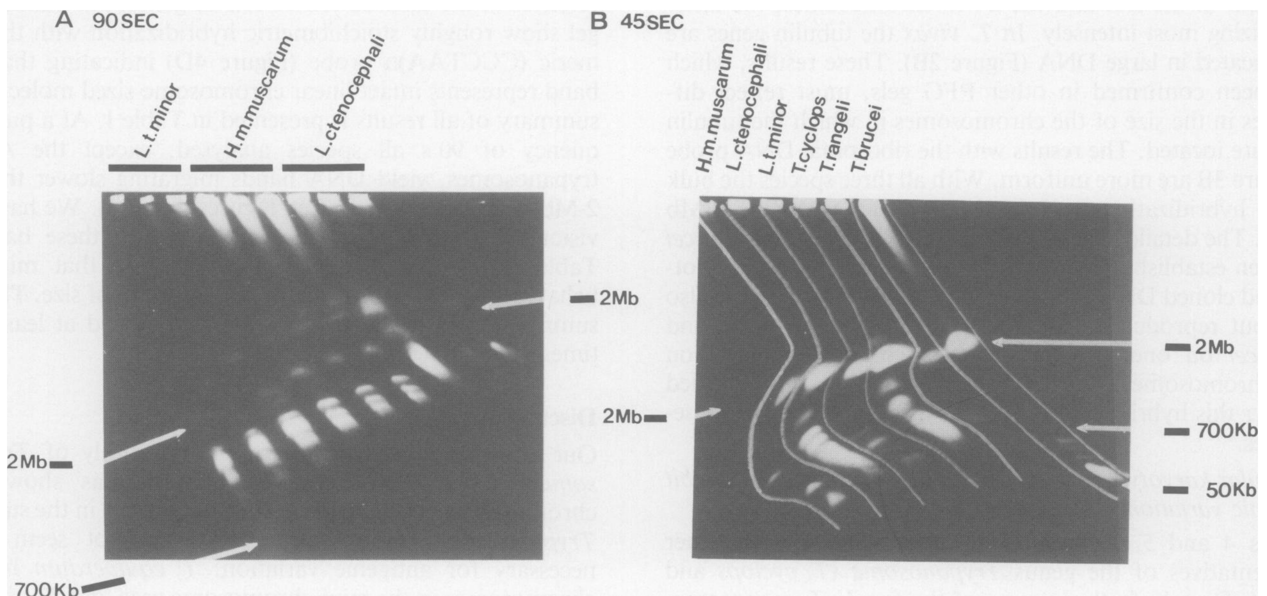

Fig. 5. PFG gel electrophoresis of Kinetoplastida at different pulse frequencies. Samples were lysed and electrophoresed as described in Materials and methods. (**A**) Electrophoresis was performed for 18 h with a pulse frequency of 90 s and (**B**) 18 h at a pulse frequency of 45 s. The strangely bent labels in **panel B** are caused by the co-migration of DNA samples with different molecular karyotypes. The migration of chromosomes within one lane is indicated with white lines.

Table I. Main features of the molecular karyograms of nine kinetoplastid species

Species	Chromosome size			Gene location								
	Range of migrating chromosomes in kb	No. of chromo-somes >700 kb[a]	No. of chromo-somes <700 kb	Mini-exon[d]			Tubulin			rDNA		
				Slot	≥2000 kb	<2000 kb	Slot	≥2000 kb	<2000 kb	Slot	≥2000 kb	<2000 kb
T. brucei	50–2000	c	~100	+			+			+	+	
T. equiperdum	50–2000	c	~10	+				+		+	+	
T. vivax	2000	c	0	+			+			+	+	
T. cyclops	700–4000[b]	>20	0		+				+	+	+	+
T. rangeili	700–4000	>20	0	+			+			+		
L. t. minor	700–4000	>20	0	ND				+	+		+	
H. m. muscarum	700–4000	>20	0		+			+		+	+	
L. ctenocephali	700–4000	>20	0	ND			+		+	+	+	
C. fasciculata[e]	700–2000	>10	0	+			ND			ND		

[a]Tentative estimates of the total number of chromosomes were obtained by adding up the number of chromosomal bands and the copy number of each band estimated by their relative intensities after ethidium staining (see Van der Ploeg *et al.*, 1984a).
[b]The extrapolation to 4000 kb is tentative (see Results).
[c]The exact number of chromosomes cannot be determined as a large fraction of the DNA remains close to the slot (see Van der Ploeg *et al.*, 1984a).
[d]Hybridization was with the synthetic 22-mer oligonucleotide which is complementary to part of the mini-exon of VSG mRNAs; hybridization conditions as described by De Lange *et al.* (1983).
[e]See Van der Ploeg *et al.*, 1984a.
ND: not done.
+: hybridization in that area.

T. brucei brucei having only few mini-chromosomes, like the single *T. equiperdum* strain analyzed here (W.C. Gibson and P. Borst, unpublished results). Nevertheless, it is likely that mini-chromosomes have evolved solely to allow a more versatile use of antigenic variation (Borst *et al.*, 1983b). Many, if not all, mini-chromosomes carry VSG genes at their ends (Van der Ploeg *et al.*, 1984a). These VSG genes are flanked downstream by long stretches of sub-telomeric and telomeric repeats (Van der Ploeg *et al.*, 1984c) and upstream by 70-bp repeats, sometimes also present in very long stretches (Van der Ploeg *et al.*, 1982; Van der Ploeg and Cornelissen, 1984). In addition, mini-chromosomes contain the 177-bp satellite DNA sequences (Sloof *et al.*, 1983a, 1983b; Van der Ploeg *et al.*, 1984a). There is no evidence of other genes in mini-chromosomes.

Why expansion of telomeric VSG gene repertoire should be advantageous to *T. brucei* is not known. It is possible that telomeric VSG genes are subject to frequent recombinations (Frasch *et al.*, 1982; Bernards *et al.*, 1983; Pays *et al.*, 1983; Van der Ploeg *et al.*, 1984c) thus allowing the organism a more extensive variation in its cell surface coats. It is also possible that the presence of many telomeric genes allows a rapid reprogramming of the order in which VSG genes are expressed in a chronic infection (Borst *et al.*, 1983b), which may be of importance for trypanosomes competing for entry into a trypanosome-infected vertebrate host. The most plausible explanation for the expansion of the mini-chromosomal repertoire in *T. brucei* is the amplification of one or a few small chromosomes. The presence of chromosomal repeats, found on all but the mini-chromosomes (unpublished results) is compatible with such descendance. Small artificial chromosomes in yeast segregate with lowered fidelity (Murray and Szostack, 1983) and if this would also apply to trypanosomes, the number of mini-chromosomes might rapidly expand under selection. The minimal size of 50 kb may be dictated by the minimal length required for any mitotic stability (Murray and Szostack, 1983).

T. brucei is considered to be diploid for its house-keeping genes (Tait, 1980; Borst *et al.*, 1982), but the analysis of chromosome translocations strongly indicates that only a haploid complement of intermediate-sized chromosomes is present (Van der Ploeg *et al.*, 1984b). The ploidy of mini-chromosomes remains undefined, because the only specific marker genes on these chromosomes – VSG genes – exchange so frequently.

Since the kinetoplastid flagellates do not condense their chromosomes, the number and size of these chromosomes cannot be determined by light microscopy of lysed metaphase cells. Several attempts have been made to analyse chromosomes in dividing cells by electron microscopy. This has led to tentative estimates of 10 chromosomes in *T. cruzi* (Solari, 1980a,1980b) and three chromosomes in *Blastocrithidia triatomae* (Solari, 1983). PFG gel electrophoresis has allowed us to get a much more precise picture. All species studied, except perhaps *T. vivax* and *Crithidia fasciculata*, have at least 20 chromosomes. The genome size of these species is relatively small; on average 0.1 pg/nucleus with a genomic complexity of 4 x 10^4 kb in *T. brucei* (Borst and Fairlamb, 1976; Borst *et al.*, 1980, 1982). This can be accounted for by 20 chromosomes with an average size of 2 Mb. Our results indicate that in general the Kinetoplastida, like fungi, have many small chromosomes rather than few larger ones. The fact that all chromosome-sized molecules in each species analyzed hybridize with the telomeric (CCCTAA)n probe, shows that telomeric sequences are conserved in this family. The fact that most of the DNA enters the gel indicates that these organisms have linear chromosomes with (CCCTAA)n ends. Larger circles do not enter these PFG gels (Schwartz and Cantor, 1984). Another point of interest is that the ribosomal genes are invariably found in large DNA. This does not conform to the situation in ciliates and slime moulds, in which the ribosomal genes are found on amplified small extra-

chromosomal elements (e.g., Herschell and Weiner, 1981; Blackburn et al., 1983).

Our analysis shows that chromosome size and composition are not highly conserved among kinetoplastid flagellates. This is exemplified by the distribution of ribosomal genes and tubulin genes. Large differences in tublin gene arrangements between the genus *Trypanosoma* (Thomashow et al., 1983; Seebeck et al., 1983) and the genus *Leishmania* (Landfear et al., 1983; Huang et al., 1984) has also been observed by Southern blot analysis. This plasticity of the kinetoplastid genome may make PFG gel analysis a useful tool in taxonomic studies, to identify sub-species indistinguishable by other means, or for the identification of extra-chromosomal elements involved in drug resistance (Peters, 1976; Coderre et al., 1983).

Materials and methods

Kinetoplastid strains

We used the following strains: (i) *T. brucei* stock 427, MITat 1.8, isolated from a sheep in Uganda and syringe passaged in laboratory rodents (Cross, 1975; Michels et al., 1984). (ii) *T. brucei*, East African Trypanosomiasis Research Organization 2340 (GUTAR 7) (Barry et al., 1983) a human isolate from Kenya. A clone passaged by syringe in mice 40 times was grown in rats for these experiments. This *rhodesiense* variant can be considered a second *T. brucei* stock (cf., Borst et al., 1981; Rickman et al., 1984). (iii) *T. equiperdum* (ATCC 30023; Hajduk and Cosgrove, 1979), a spontaneously dyskinetoplastic trypanosome clone, grown in rats for these experiments. (iv) *T. vivax* (Y58) (Leeflang et al., 1976), isolated from a cow in Nigeria; a clone passaged by syringe 61 times in mice, then grown in sub-lethally irradiated mice (600 rads). (v) *T. rangeli* (R 1306; Ellis et al., 1980), isolated from a dog in Colombia; grown in minimal essential medium supplemented with 20% heat-inactivated foetal calf serum at 26°C. (vi) *T. cyclops* (Weinman, 1972), obtained from D. Weinman, Yale University, and isolated from *Macaca nemestrina*; grown in SM medium (Cunningham, 1977) supplemented with 10% heat-inactivated foetal calf serum. (vii) *Herpetomonas muscarum muscarum* (ATCC 30260) (Rogers and Wallace, 1971), isolated from the house fly, *Musca domestica*; maintained in Liver Infusion Tryptose medium supplemented with 10% heat-inactivated foetal calf serum (Camargo, 1964) at 26°C. (viii) *Leptomonas ctenocephali* (LV117); obtained from the Liverpool School of Tropical Medicine and isolated from the flea *Xenopsylla* sp.; grown in Liver Infusion Tryptose medium. (ix) *Leishmania tropica minor* was obtained from Dr. D. Hart, Institute of Cellular and Molecular Pathology, Brussels; grown in SDM medium supplemented with 10% heat-inactivated foetal calf serum at 26°C (Brün and Schönenberger, 1979).

Trypanosomatids were purified free from blood elements as described previously (Fairlamb et al., 1978).

PFG gel electrophoresis

Lysis of trypanosomes and PFG gel electrophoresis were performed essentially as described by Schwartz and Cantor (1984) and Van der Ploeg et al. (1984a). Modifications introduced were the use of a 1% agarose gel at a temperature of 20°C at 17.5 V/cm in the North/South and 6.15 V/cm in the West/East direction. This gave improved resolution and separation of chromosomes in the gel. Two technical problems remain. Firstly, large DNA molecules loaded in the North-West corner of the gel tend to undergo more shear degradation than molecules loaded in slots near the middle of the gel. Secondly, large DNA molecules loaded in the North-West corner tend to enter the gel less effectively than molecules loaded in the middle (compare for instance lanes 1 – 3 of Figure 3A with lanes 6 – 12 of the same Figure). Both effects are due to the inhomogeneous electrical field (see also Van der Ploeg et al., 1984a, 1984b).

The mol. wts. determined with PFG gel electrophoresis are tentative. No systematic analysis has been made of the possible effects of DNA sequence on mobility in this system and no completely reliable DNA markers are available for calibration of gels in the size range above 200 kb. We have previously calibrated the approximate sizes of the chromosome-sized DNA molecules of *T. brucei*, strain 427, by co-migration with yeast chromosomes and phage DNAs (Van der Ploeg et al., 1984a). We have used the *T. brucei* 427 DNA molecules as size markers in the experiments presented here.

Southern blotting and hybridization

Sub-probes of recombinant plasmids were isolated by preparative agarose electrophoresis of the appropriate restriction endonuclease digests and purification of the fragments from low-melting agarose by diethylaminoethyl-column chromatography (Smith, 1980). The fragments were then labeled by nick-translation (Rigby et al., 1977). We used the following probes: 1, a partial *Pst*I fragment of 18 kb containing a complete repeat of the rRNA transcription unit of *T. brucei* (see Hasan et al., 1982), cloned in pBR 328 (T. Berkvens and L.H.T. Van der Ploeg, unpublished results); 2, the α- and β-tubulin genes on a 3.7-kb *Hin*dIII fragment cloned by Thomashow et al. (1983); 3, the (CCCTAA)n telomere-specific repeat of *T. brucei*, cloned in plasmid pUR 222 (clone pT6; Van der Ploeg et al., 1984c). Transfer of DNA to nitrocellulose filters was performed as described by Southern (1975). The gels were pre-incubated with 0.25 M HCl for 40 min prior to the alkali denaturation step to reduce the mol. wt. of the fragments. Hybridization of the filters at 65°C in 3 x SSC [1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0)] was performed as described by Jeffreys and Flavell (1977), with addition of 10% dextran sulphate (Van der Ploeg et al., 1982). Post-hybridizational washes to remove aspecifically-bound probe were carried out for 2 h at 65°C with several changes of 3 x SSC. At this point the filters were either autoradiographed directly or – as indicated in the text – washing was continued with a lower salt concentration to melt off imperfectly matched hybrids. To achieve this we lowered the salt concentration stepwise (1 x, 0.3 x, 0.1 x SSC) each step taking 30 min at 65°C.

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