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VARIATIONS IN MAXI-CIRCLE AND MINI-CIRCLE SEQUENCES IN KINETOPLAST DNAs FROM DIFFERENT *TRYPANOSOMA BRUCEI* STRAINS

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Summary

We have compared a total of 30 recognition sites for eight restriction endonucleases on the 20-kilobase-pair maxi-circle of kinetoplast DNAs from five different *Trypanosoma brucei* strains. In addition to three polymorphic sites we have found a 5 kilobase-pair region that is not cleaved by any of the eight enzymes and that varies in size over 1 kilobase pair in the strains analysed.

Mini-circles from these five strains, digested with endonuclease *TaqI* or *MboII*, yield very complex fragment patterns, showing that extensive mini-circle sequence heterogeneity is a common characteristic of these *T. brucei* strains. The size distribution of mini-circle fragments in these digests was identical for different clones of the 427 strain, but very different for mini-circles from different strains.

These results show that maxi-circle sequence is conserved, whereas mini-circle sequence is not. Restriction digests of maxi-circles could be useful in determining how closely two *Trypanosoma* strains are related, whereas mini-circle digests can serve as sensitive tags for individual strains.

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Abbreviations: kDNA, kinetoplast DNA; VSG, variant surface glycoprotein.

Introduction

The kinetoplast DNA (kDNA) of trypanosomes is an unusual form of mtDNA that consists of networks of about 10^4 catenated circles. In most trypanosome species two types of circles are present in the network, mini-circles [1–3] and maxi-circles [4–6]. The mini-circles take up 90% or more of the network mass and they determine its size and shape [7]. They are heterogeneous in sequence [5,8,9] and not transcribed in two [10,13] of the three [11] species studied; their function is not known. Maxi-circles, in contrast, lack sequence heterogeneity [6,7,10], they are transcribed [12,13] and they are probably the counterpart of the mtDNA of other organisms (see Refs. 7, 10 and 14).

Previous work on *T. brucei* kDNA has shown that it contains 0.9 kilobase-pair mini-circles and 20-kilobase-pair maxi-circles [15–19]. Optical renaturation studies suggested that the mini-circles consist of at least 100 different sequence classes [20]. The evolution of these sequences appeared to be rapid: only partial cross-hybridization was found between the kDNAs of two *T. brucei* strains [20]. Differences in size were also found between the maxi-circles of both strains [17].

The construction of a map of the maxi-circle of *T. brucei* strain 427 [18,19] and the identification of restriction endonucleases that cut a substantial fraction of the mini-circles more than once [7,10,17] has made it possible to analyse the sequence evolution of maxi-circles and mini-circles separately and in more detail. The results of this analysis, briefly summarized in a recent review [7] and at symposia [10,18], are presented here.

Materials and Methods

Source and designation of trypanosome strains

Trypanosoma brucei brucei, 427 was from a sheep in South-East Uganda [23]. Clones 427-60 (MITat 1.1), 427-117 (MITat 1.4) and 427-118 (MITat 1.5) were obtained from Dr. G.A.M. Cross (The Wellcome Research Laboratories, Department of Immunochemistry, Beckenham, U.K.). These clones express different variant surface glycoproteins (VSGs) (see Ref. 24). In previous papers we have erroneously referred to the 427-60 clone as EATRO 427 (see Refs. 10, 17, 19). We do not know whether this clone is still only expressing VSG 60.

Trypanosoma brucei, ssp. 31, was obtained from Professor D. Zwart (Institute of Tropical and Protozoological Diseases, State University, Utrecht, The Netherlands). This is an old laboratory strain, probably from East Africa, but no complete records are available. Although this strain contains kDNA, which is normal by all criteria available [10], the mitochondrial ATPase is insensitive to oligomycin (Dr. F.R. Opperdoes, International Institute of Cellular and Molecular Pathology, Brussels, Belgium; personal communication), and attempts to grow it in culture or in tsetse flies have been unsuccessful (Dr. D.A. Evans, London School of Hygiene and Tropical Medicine, London, U.K.; personal communication). It is, therefore, classified as K^+ , I^- (see Ref. 25). By maxi-circle sequence analysis (see Results) and mapping of restriction endonuclease

restriction sites around nuclear genes for variant surface glycoproteins (Frasch, A.C.C., Bernards, A. and Borst, P., unpublished results) this strain is very similar to *T. brucei* EATRO 839, which cannot be distinguished from a known *T. brucei rhodesiense* strain (see below).

Trypanosoma brucei brucei, EATRO 1125, was obtained from Professor J. Mortelmans (Institute of Tropical Medicine 'Prins Leopold', Antwerp, Belgium) via Dr. M. Steinert (Free University, Rhode-St.-Genèse, Belgium). The immediate parent reference number is ITMAS 251174B/AnTat8/Antademe I.

Trypanosoma brucei brucei, EATRO 839, was from a zebu in Alego. The strain used is from Professor D. Zwart. EATRO 839 is identical to LUMP 227 (see Ref. 26). Although samples of this strain were non-infective to one human volunteer and sensitive to lysis by human serum [21,22], it is indistinguishable by isoenzyme analysis [26] and maxi-circle sequence analysis (Borst, P., Fase-Fowler, F. and Gibson, W.C., unpublished results) from a known *T. brucei rhodesiense* strain, LUMP 1196.

Trypanosoma brucei brucei, LUMP 1027, was obtained from Dr. D.A. Evans. Probably this is one of the dyskinetoplastic strains derived by Stuart (see Ref. 27), but this is not certain (Evans, D.A., personal communication).

Trypanosoma brucei brucei, LUMP 127, clone 7, was obtained from Dr. F.R. Opperdoes. The primary isolate was obtained from *Glossina pallidipes* at EATRO in 1960; the ETat group of stabilates were also derived from this isolate. Although this strain contains kDNA, which is normal by all criteria available [10], the mitochondrial ATPase is insensitive to oligomycin and the strain does not grow in culture (Dr. R.F. Steiger, International Institute for Cellular and Molecular Pathology, Brussels, Belgium; personal communication). Subclones of this strain appear to have lost sensitivity to the lytic action of human blood and this strain may in fact be a *Trypanosoma rhodesiense* strain (Opperdoes, F.R., personal communication).

Growth and isolation of trypanosomes

Trypanosomes were grown in rats and kDNA was isolated as described [17, 19].

Endonuclease digestion and gel electrophoresis of DNA

Restriction endonuclease digestion was carried out under standard conditions as described [19]. Horizontal agarose gels (21 × 21 × 0.5 cm) were run with buffer containing 0.5 µg ethidium bromide per ml as described [19], but using slots of 1 mm width instead of 2 mm to increase resolution. Two conditions were used for electrophoresis: 'low-voltage' gels were run overnight in 40 mM Tris-HCl/1 mM sodium EDTA/20 mM sodium acetate (pH 7.7) at 25 V and 40 mA. 'High-voltage' gels were run for 2–3 h in 90 mM Tris-borate/2.5 mM sodium EDTA (pH 8.3) [28] at 90 mA and unknown voltage (over 100 V) with vigorous recirculation of buffer.

Molecular weight markers for gel calibration

The following DNAs and DNA fragments were used: B, bacteriophage ϕ 29 DNA plus an *Eco*RI digest of this DNA (see Ref. 29); D, bacteriophage PM2 DNA digested with *Hind*III (see Ref. 30).

In some experiments the linearized mini-circle of *T. brucei*, which is 0.9 kilobase pairs in dilute gels [19] was used as an additional marker.

Source of endonucleases

Endonucleases *Pst*I [31], *Hap*II [32], *Bgl*I + *Bgl*II [31], *Hind*III [33], *Sst*I [34], *Xba*I [34] and *Hha*I [35] were purified by published procedures. Endonucleases *Eco*RI and *Bgl*II were bought from Boehringer Mannheim and endonucleases *Hae*III, *Bgl*II and *Mbo*I from New England Biolabs. Endonuclease *Sau*96I (see Ref. 36) was a gift from Dr. J.S. Sussenbach, State University, Utrecht.

Nomenclature of restriction fragments

Short-hand abbreviations (see Ref. 37) for the enzymes used are: Ps for *Pst*I, Y for *Hap*II, R for *Eco*RI, G for *Bgl*II, D for *Hind*III, L for *Hha*I, Ss for *Sst*I, Xb for *Xba*I, E for *Hae*III, U for *Sau*96I, B for *Bam*HI and Q for *Taq*I. The sites at each end of a fragment are identified using these short-hand abbreviations.

DNA-DNA hybridization experiments

Blotting of electrophoretically separated DNA fragments onto nitrocellulose and hybridization of the blots with nick-translated ³²P-labelled DNA of lambda-gt.WES containing the *Eco*-2 fragment (bordered by sites R1 and R2 in Fig. 2) of the *T. brucei* 427-60 maxi-circle [38] was done as described [39]. The cloned DNA was kindly provided by Dr. J. Davison (International Institute of Cellular and Molecular Pathology, Brussels, Belgium).

Other materials

Other materials were the same as in Ref. 19.

Map construction

We have refined our published maxi-circle map [19] for *T. brucei* 427-60 on several points. The two *Bgl*I sites have turned out to be *Bgl*II sites; our home-made *Bgl*I preparation was contaminated with *Bgl*II, and *Bgl*I + *Bgl*II gives the same fragments as *Bgl*II alone. From indirect evidence we had inferred the presence of three small *Xba*I fragments, which could not be seen in gels because they co-migrated with mini-circles. Improved gel technology has led to the detection of two small *Xba*I fragments, 1.5 and 1.3 kilobase pairs, respectively, which together account for the missing maxi-circle segment. This confirms the results of Stuart for *T. brucei* 164. The order of the two fragments given in Fig. 2 is taken from Stuart [40]. Following Stuart's report [40] of a single *Bam*HI site in the maxi-circle of strain 164, we found a single *Bam*HI site at the corresponding position in the maxi-circle of strain 427, in contrast to our previous report [19] that *Bam*HI does not cut the 427 maxi-circle. The position of the *Bam*HI site was precisely determined in *Bam*HI × *Eco*RI and *Bam*HI × *Hind*III double digests (not shown).

On circumstantial evidence we have previously postulated [19] the presence of two adjacent small *Hha*I fragments — LL4 and LL5 — in 427 maxi-circles, lying between sites L1 and L3 in Fig. 2. LL5 of 0.4 kilobase pair overlaps frag-

ment RR2 (fragment bordered by sites R1 and R2) and indeed such a small fragment faintly hybridized with a labelled RR2 probe in lanes 2–4 and 9 of Fig. 3. This fragment was absent in lanes 5–8 and the only hybridization observed was with fragment LL2 (bordered by sites L5 and L1), which, therefore, includes LL5 in these strains (only results for 427-118 and 31 are shown in lanes 10 and 11). Direct evidence for the 0.9 kilobase pair fragment LL4 is presented in lane 12 of Fig. 3, which shows a partial digest of the 127 maxi-circle with partial digestion products differing in size by about 1 kilobase pair.

A partial *TaqI* map (see Fig. 2) of the maxi-circle of strain 427 was made by redigestion of *TaqI* digests with a second restriction endonuclease and by hybridization of *TaqI* digests with the cloned RR2 (*Eco*-2) or RR3 (*Eco*-3) fragments [38,39]. Fragment QQ1 (bordered by sites Q5 and Q6 in Fig. 2) does not hybridize (see Ref. 39) with either RR2 (*Eco*-2) or RR3 (*Eco*-3), it is not cut by *EcoRI*, whereas *HindIII* cuts off 0.5 kilobase pairs from this fragment and its counterpart in strain 127 (not shown). Fragments QQ2, QQ3 and QQ4 hybridize [39] with RR2 and not with RR3; *HindIII* cuts QQ4 and *EcoRI* cuts QQ3 (results not shown; see Fig. 2). A partial digestion product 0.8 kilobase pairs longer than QQ1 is seen in partial *TaqI* digests (not shown). This could be the fragment bordered by sites Q4 and Q5; this fragment is not visible in digests, because it falls under the mini-circle fragments. The 0.4 kilobase pair fragment between Q6 and Q7 is inferred from the size difference between QQ1 in different strains, which is 0.4 kilobase pairs less than observed for other fragments which overlie this region (see Fig. 4). Since the 0.4 kilobase-pair difference is conserved in the *HindIII* × *TaqI* digest (not shown), this small fragment must be present on the right-hand side of QQ1 in Fig. 2, as drawn. Attempts to detect this fragment in partial digests have been unsuccessful and we do not have a suitable probe for its detection by hybridization because it lies within fragment RR1 (*Eco*-1), which has resisted cloning in *Escherichia coli* [38].

Results

Comparison of maxi-circle digests on gels

To obtain information on the degree of sequence homology between the maxi-circles from different *T. brucei* strains, kDNAs from five strains were digested with restriction endonucleases and run side-by-side on gels. The *EcoRI* and *HindIII* digests are presented in Fig. 1. Strain 427-60 is the one studied previously in this laboratory; 427-117 is another clone of the 427 strain (see Materials and Methods). The other strains are unrelated to 427 as far as we know (see Materials and Methods and Table I). In both digests two of the three maxi-circle bands are identical in all strains and only the bands with the lowest mobility (RR1 and DD1) vary. The map of the maxi-circle fragments from 427, presented in Fig. 2, shows that the RR1 (between R2 and R3) and DD1 (between D2 and D3) fragments share a common region of 6.5 kilobase pairs. Apparently, this segment varies in size in different strains.

The digests shown in Figs. 3–5 confirm and extend this conclusion. The fragments with the lowest mobility in the *HhaI* digest (Fig. 3), the *TaqI* digest (Fig. 4B) and the *MboI* digest (Fig. 5; only results for three strains shown) show the same strain-dependent variations in mobility observed in the digests

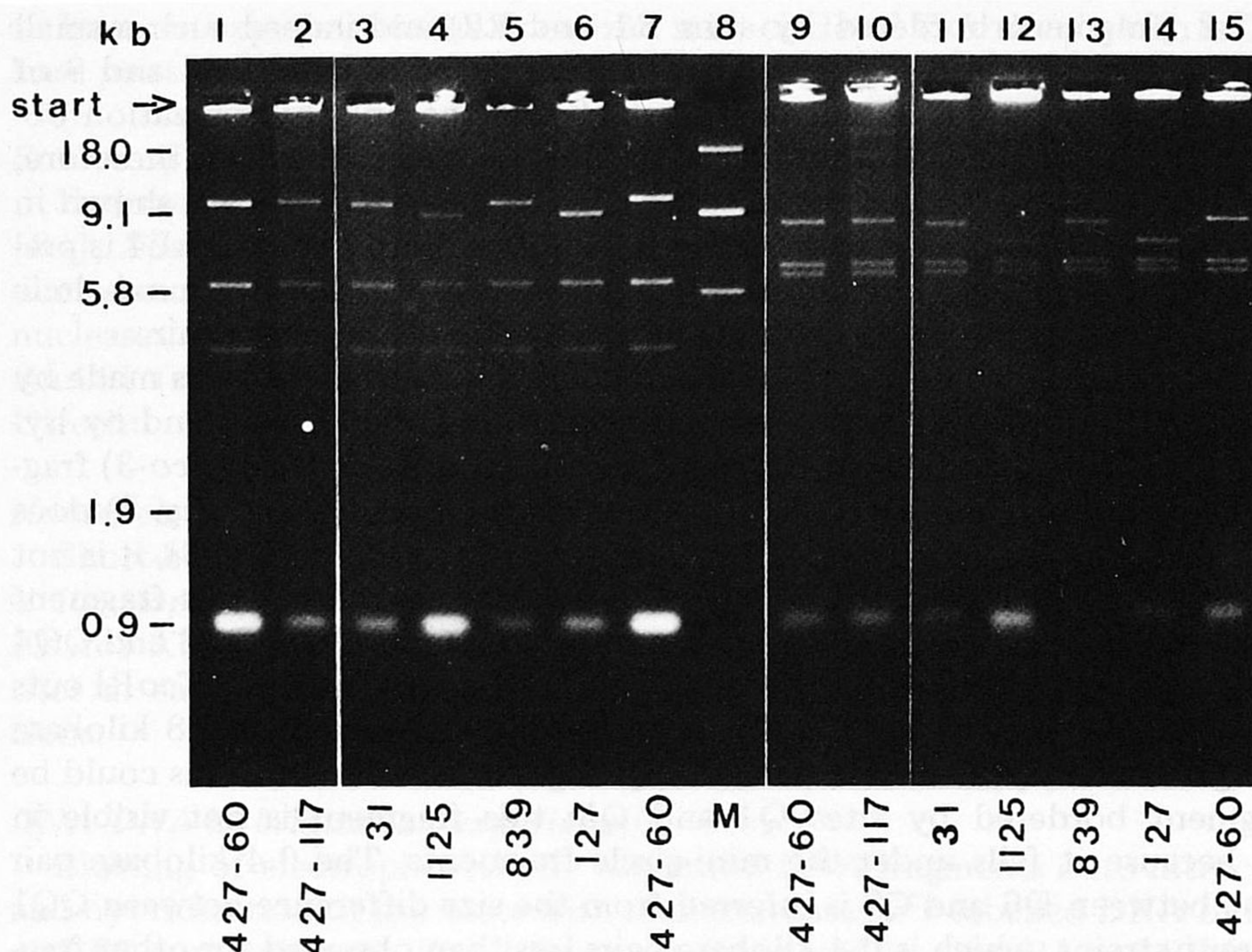


Fig. 1. A comparison of maxi-circle fragments from various *T. brucei* strains derived from kDNA by cleavage with *EcoRI* or *HindIII*. The kDNAs were digested either with *EcoRI* (lanes 1–7) or *HindIII* (lanes 9–15), 0.15- μ g or 0.2- μ g samples were electrophoresed in a 0.7% agarose 'low-voltage' gel and the ethidium-stained gel was photographed. Lane 8 contains marker B. The strain from which each kDNA was isolated is indicated underneath the lanes. kb, kilobase pairs.

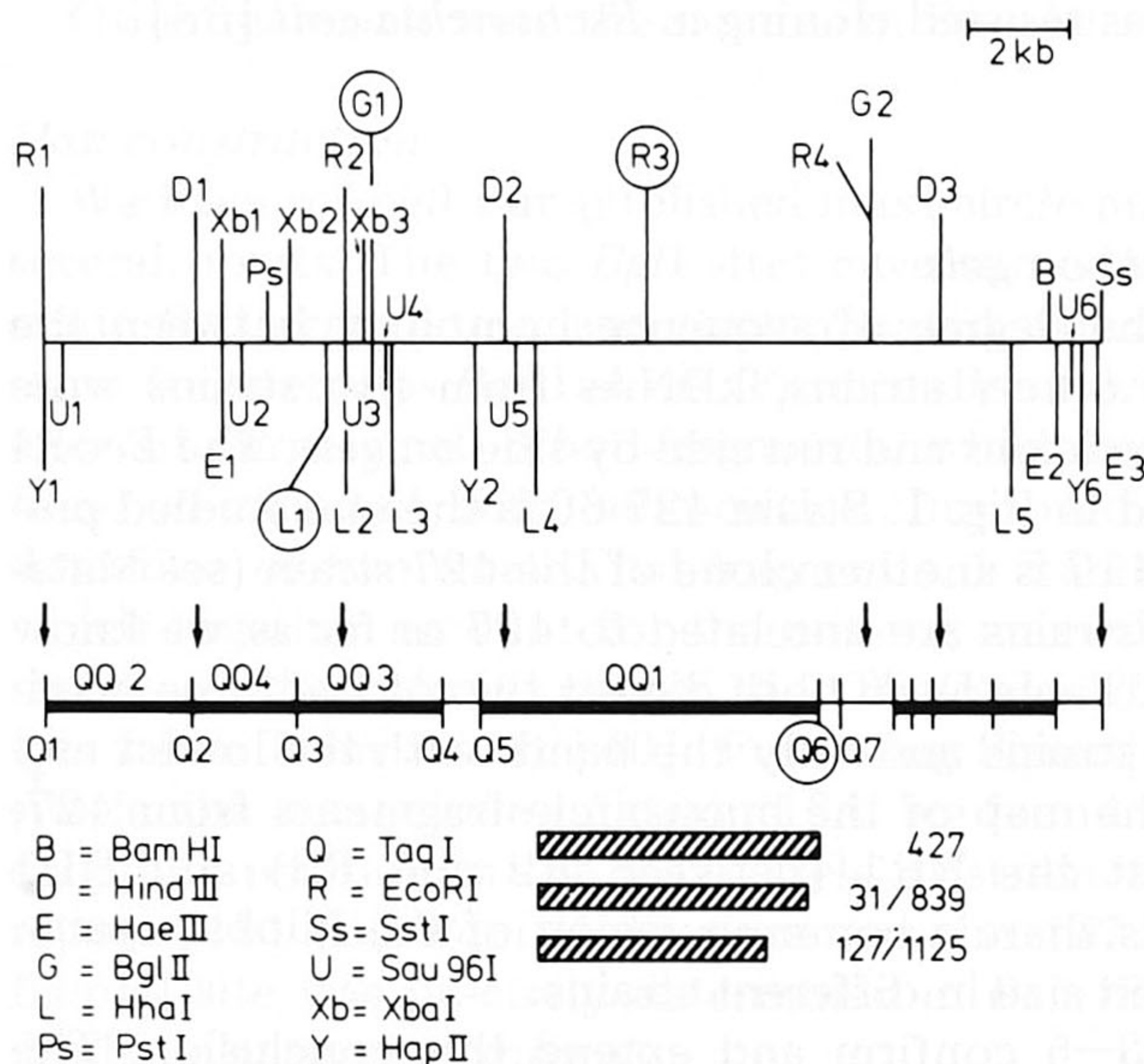


Fig. 2. The linearized physical map of the maxi-circle from *T. brucei* kDNA. The upper part shows our published map [19] for strain 427-60 with the corrections and additions specified in Materials and Methods. The length is 20.5 kilobase pairs. The middle part presents a partial *TaqI* map. The arrows indicate the positions of *EcoRI* and *HindIII* sites. There are many *TaqI* sites between Q7 and Q1, but only part of these have been mapped. The lower part gives the size of the 'variable region' in the different strains. Polymorphic sites are circled. See Materials and Methods under Map construction for further details. kb, kilobase pairs.

TABLE I
TRYPANOSOMA BRUCEI STOCKS USED

I refers to the ability to make functional mitochondria, i.e. I⁺ strains are able to grow in culture and multiply in the insect vector, I⁻ strains are not [25]. K refers to the presence (K⁺) or absence (K⁻) of kDNA networks [25]. n.d., not determined.

Full name	Abbreviated name	Characteristics	
		I	K
427-60 (MITat 1.1)	427-60	I ⁺	K ⁺
427-117 (MITat 1.4)	427-117	n.d.	K ⁺
427-118 (MITat 1.5)	427-118	n.d.	K ⁺
31	31	I ⁻	K ⁺
EATRO 1125	1125	I ⁺	K ⁺
EATRO 839	839	I ⁺	K ⁺
LUMP 1027	1027	I ⁻	K ⁻
LUMP 127	127	I ⁻	K ⁺

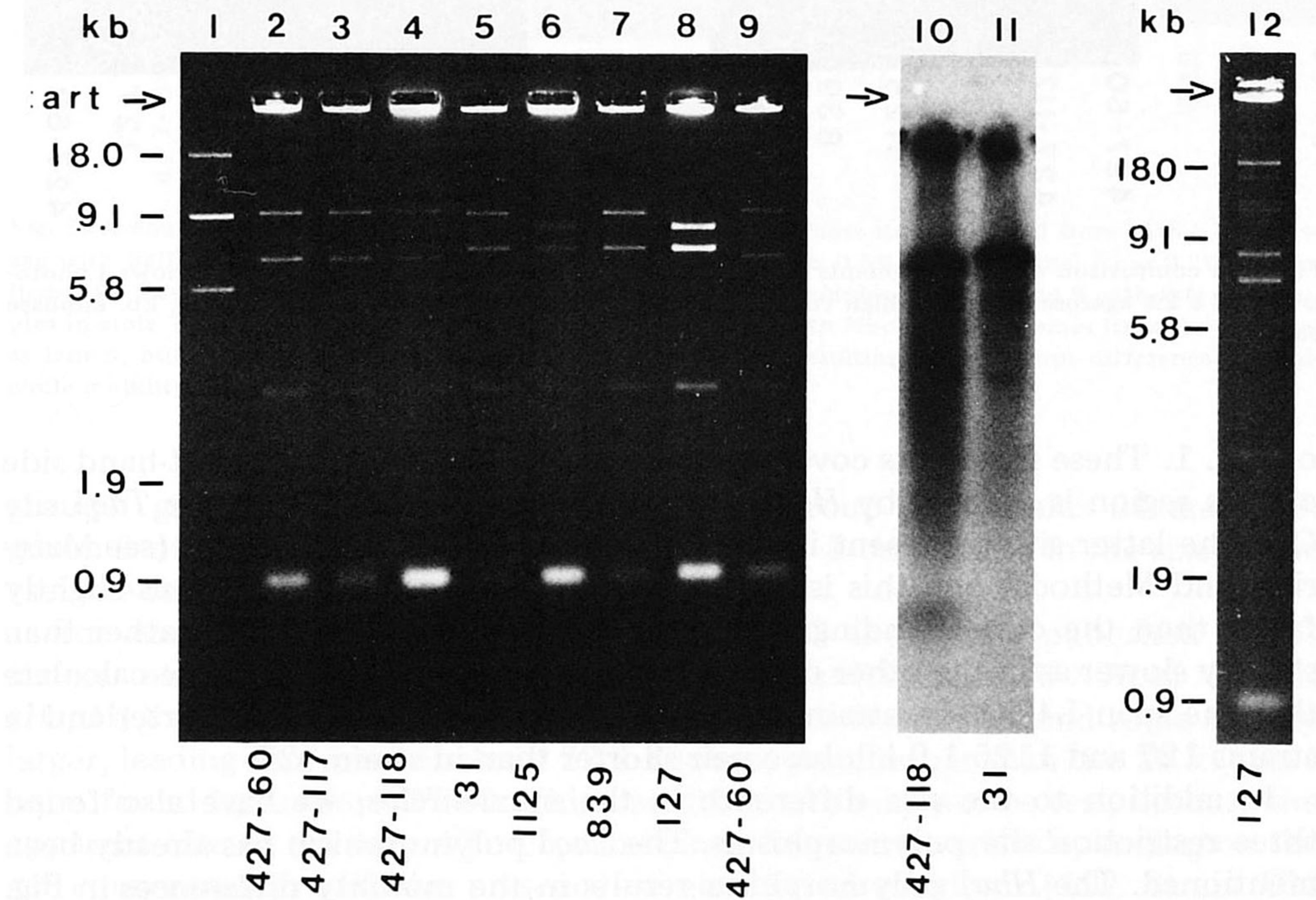


Fig. 3. A comparison of maxi-circle fragments from various *T. brucei* strains derived from kDNA by cleavage with endonuclease *HhaI*. Conditions as in Fig. 1. Lane 1 contains marker set B. The DNA in lanes 1–9 was transferred to a nitrocellulose filter and hybridized with labelled recombinant phage lambda DNA containing the RR2 (*Eco*-2) fragment, as described in Materials and Methods. The part of the autoradiogram corresponding to lanes 4 and 5 is reproduced in lanes 10 and 11. (There was a slight contamination with *Eco*RI-digested phage lambda DNA in all slots of this gel, leading to faint phage lambda bands in addition to the *HhaI* fragments of the maxi-circle that hybridize with the labelled probe.) Lane 12 contains a partial *HhaI* digestion of 127 kDNA (taken from a different experiment). kb, kilobase pairs.

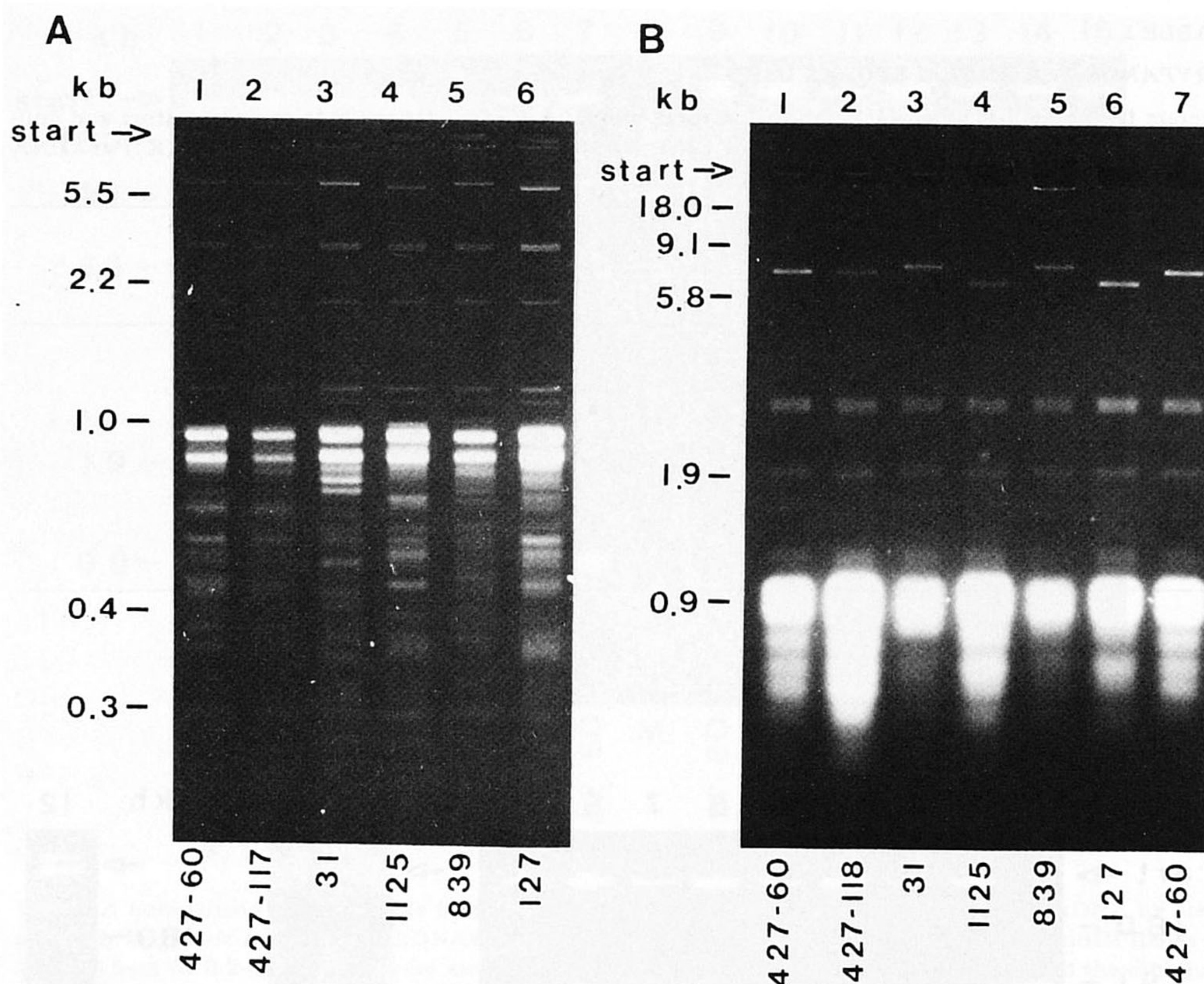


Fig. 4. A comparison of *TaqI* fragments of kDNAs from various *T. brucei* strains. Panel A shows a photograph of a 2% agarose gel run at high voltage, panel B a 1% agarose gel run at low voltage. kb, kilobase pairs.

of Fig. 1. These fragments cover the same region of the map. The left-hand side of this region is defined by *HhaI* site L4, the right-hand side by the *TaqI* site Q6. The latter site is present in 427-60 but not in the other strains (see Materials and Methods) and this is why the QQ1 fragment of 427-60 runs slightly faster than the corresponding fragments from strains 31 and 839 rather than slightly slower as in the other digests. From these and other digests we calculate that the span L4—Q6 in strains 31 and 839 is 0.2 kilobase pairs shorter and in strains 127 and 1125 1.0 kilobase pair shorter than in strain 427.

In addition to the size difference in the maxi-circles, we have also found three restriction site polymorphisms. The *TaqI* polymorphism has already been mentioned. The *HhaI* polymorphism results in the mobility differences in Fig. 3 of fragment LL2 and the concomitant presence or absence of a small 0.4 kilobase pair fragment only detected by hybridization (see Fig. 3, lane 10). The absence of a second *BglII* site in strains 31, 127 and 1125 is shown in Fig. 5. The map position of these polymorphic sites is indicated in Fig. 2 (circled sites) and discussed in more detail in Materials and Methods under Map construction. Table II lists the number of restriction sites screened for each strain, Table III summarizes the differences found. It is clear that our strains fall in three

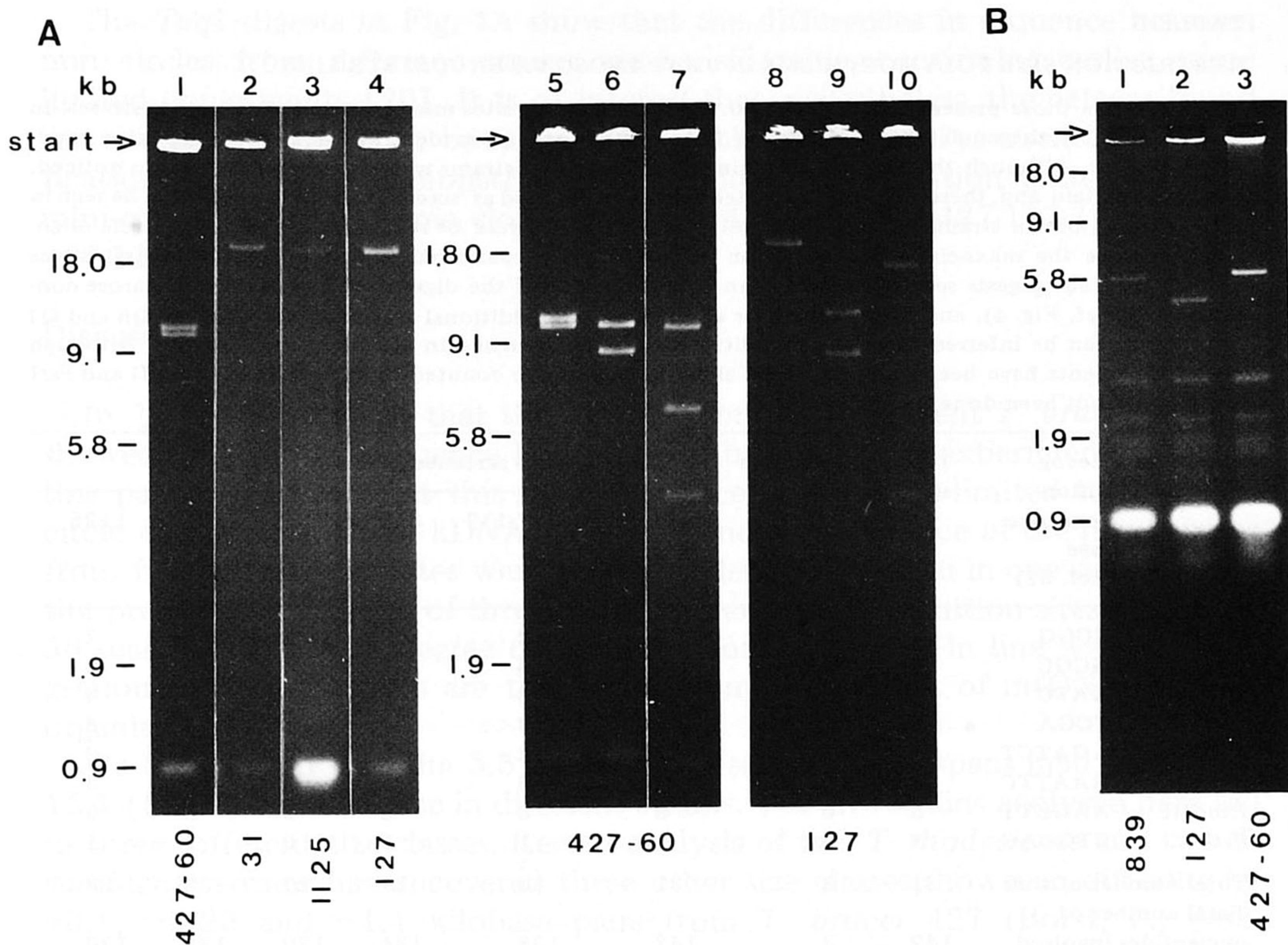


Fig. 5. A comparison of maxi-circle fragments from various *T. brucei* strains derived from kDNA by cleavage with *Bgl*III or *Mbo*I. Digests were run at low voltage through 0.5% agarose (panel A) or 0.7% agarose (panel B). Panel A, all samples digested with *Bgl*III; in addition, samples in slots 6 and 9 with *Pst*I and samples in slots 7 and 10 with *Sst*I. Panel B, all samples digested with *Mbo*I. Lane 7 comes from the same gel as lane 6, but several lanes between 6 and 7 were taken out, resulting in the abrupt difference in mini-circle mobility. kb, kilobase pairs.

groups: group 1 consists of the 427 clones; group 2 of strains 31 and 839; group 3 of strains 127 and 1125. Groups 2 and 3 only differ in the size of the variable region.

For comparison, we have added to Table III the results published for *T. brucei* strain 164 by Stuart [40]. His *Eco*RI \times *Hind*III \times *Xba*I \times *Hpa*II (*Hap*II) \times *Bam*HI map is identical to ours, but his fragments tend to be slightly larger, leading to a maxi-circle of 22.2 kilobase pairs rather than the 20 kilobase pairs found by us for 427-60. This size difference may not be real, because the apparent size of the very AT-rich maxi-circle fragments varies depending on the gel concentration and the presence or absence of ethidium [19]. Strain 164 belongs to a fourth group of *T. brucei* strains, however, because it has *Eco*RI site 3. This site is lacking in our five *T. brucei* strains but we have recently found it in a *T. rhodesiense* strain at the same position as in *T. brucei*, strain 164 (Borst, P., Fase-Fowler, F. and Gibson, W.C., unpublished results).

Comparison of mini-circle digests on gels

The sequence heterogeneity of the mini-circles of *T. brucei* results in incom-

TABLE II
RESTRICTION SITE POLYMORPHISMS IN *TRYPANOSOMA BRUCEI* STRAINS

Total sites are those present in strain 427-60. Variable sites are sites missing in one of the other strains. In the case of *HapII*, the small 0.45 kilobase pair fragment has only been identified in a partial digestion product in 427-60. Although the loss of site Y3 in any of the other strains would probably have been noticed, this is not certain and, therefore, only two sites have been counted as 'screened'. Six fragments can be seen in *MboI* digests, but in strains 31 and 1125 only part of these could be resolved from the mini-circle oligomers, because the maxi-circle concentration in the kDNA preparations available from these strains was low. In the *TaqI* digests seven fragments can be clearly seen if the digests are run in several agarose concentrations (cf. Fig. 4), and the presence or absence of one additional fragment bordered by Q6 and Q7 (see Fig. 2) can be inferred from the mobility of QQ1, as discussed in Materials and Methods. Although not all fragments have been mapped, these eight fragments are counted as eight sites. The *BglII* and *PstI* digests have not been done for strain 839.

Endo-nuclease	Recognition sequence (see Ref. 52)	Total sites	Variable sites	Number of sites screened in strain					
				427-60	427-117	31	839	127	1125
<i>HapII</i>	CCGG	3	0	3	2	2	2	2	2
<i>HhaI</i>	GCGC	5	1	5	5	5	5	5	5
<i>MboI</i>	GATC	6	0	6	6	5	6	6	4
<i>TaqI</i>	TCGA	8	1	8	8	8	8	8	8
<i>BglII</i>	AGATCT	2	1	2	2	2	0	2	2
<i>EcoRI</i>	GAATTC	3	0	3	3	3	3	3	3
<i>HindIII</i>	AAGCTT	3	0	3	3	3	3	3	3
<i>PstI</i>	CTGCAG	1	0	1	1	1	0	1	1
Total number of sites		31	3	31	30	29	27	30	28
Total number of nucleotides involved		142	3	142	138	134	120	138	130

plete digestion of the networks with most of the restriction endonucleases available. As briefly mentioned previously [17], however, *TaqI* appears to cut more than 95% of the network (Fig. 4) and the same is found for *MboII* (not shown).

TABLE III
SEQUENCE COMPARISON OF THE KINETOPLAST MAXI-CIRCLES FROM DIFFERENT *TRYPANOSOMA BRUCEI* STRAINS

The 'variable region' lies between map units 9.6 and 15.1 in Fig. 2; x - 0.2 means that the 'variable region' is 0.2 kilobase pairs shorter than in strain 427-60. n.d., not determined.

<i>T. brucei</i> strain	Polymorphic restriction site				Size 'variable region' in kilobase pairs
	<i>HhaI</i> (L1)	<i>BglII</i> (G1)	<i>EcoRI</i> (R3)	<i>TaqI</i> (Q6)	
427-60	+	+	—	+	x
427-117	+	n.d.	—	+	x
31	—	—	—	—	x - 0.2
839	—	n.d.	—	—	x - 0.2
127	—	—	—	—	x - 1.0
1125	—	—	—	—	x - 1.0
164 *	n.d.	n.d.	+	n.d.	

* Data taken from Ref. 40.

The *TaqI* digests in Fig. 4A show that the differences in sequence between mini-circles from different strains are considerable, confirming earlier, more limited experiments [20]. It is of interest that, nevertheless, the pattern found for one strain appears to be sufficiently stable to be useful as a sensitive identification tag. This is demonstrated by the identical digestion patterns of the mini-circles from the three clones of strain 427, 427-60, 427-117 (Fig. 4) and 427-118 (not shown).

Discussion

In 1976 we observed that the kDNAs from two different *T. brucei* isolates showed only partial sequence homology in hybridization experiments [20]. In this paper we show that this rapid sequence evolution is limited to the mini-circle component of the kDNA. The differences in sequence of the maxi-circles from five different isolates were limited to length variation in one segment and the presence or absence of three restriction enzyme recognition sites out of the 30 scanned. This high degree of sequence conservation is in line with the suggestion that maxi-circles are the trypanosome equivalent of mtDNA in other organisms.

We have found that the 5.5 kilobase-pair segment that spans map units 9.6–15.1 (Fig. 2) varies in size in different strains. The five strains analysed here fall in three different size classes. Recent analysis of two *T. rhodesiense* and two *T. gambiense* strains has uncovered three other size classes, however, differing by +0.1, –1.25 and –1.4 kilobase pairs from *T. brucei* 427 (Borst, P., Fase-Fowler, F. and Gibson, W.C., unpublished results). This shows that this 'variable region' is a hot-spot for insertions/deletions. The size differences look rather haphazard and there is no indication for a basic insertion sequence which may be inserted several times.

There are two precedents for substantial size differences between closely related mtDNAs. In the yeast *Saccharomyces*, mtDNAs from different strains differ by 1–3 kilobase-pair insertions/deletions. These insertions/deletions are found in at least three widely separated positions in the mtDNA; they are of fixed size; the insertions have about the same mol% G + C as the rest of the DNA, and at least three of these insertions have been shown to be intervening sequences (introns) in structural genes [41]. The other precedent is the variable size of *Drosophila* mtDNA [42–44]. In this case the size variation is limited to one very AT-rich segment in the DNA. Clearly, the size variation in the maxi-circle of *Trypanosoma* resembles more that seen in *Drosophila* than that of yeast mtDNA. Since the 'variable region' of the maxi-circle is remarkably low in recognition sites for restriction enzymes, it may also be AT-rich, as pointed out before [19]. Attempts to characterize further the size differences in this region by DNA heteroduplex analysis have failed thus far due to multiple nicks in our purified maxi-circle preparations.

The usefulness of the restriction analysis of mtDNA for determining phylogenetic relationships among co-specific organisms has recently been stressed by Avise et al. [45,46] and Brown and co-workers [47,48]. The sequence evolution of mtDNA in higher vertebrates appears to be faster than that of nuclear single-copy DNA [45–50] and the analysis of mtDNA is, therefore, especially

informative when closely related organisms are compared. In mtDNAs from 21 human individuals of diverse racial background and country of origin, Brown and Goodman [48] detected only two polymorphic sites in 64 checked and they calculated a diversity for this mtDNA of 0.002 per nucleotide. A low intra-species variability was also observed in a limited series of *Drosophila* mtDNA samples by Shah and Langley [44]. For wild mice (*Peromyscus* species) and pocket gophers (*Geomys pinetus*) the difference between animals caught in different American locations was in the order of 0.01 per nucleotide [45,46]. A matrix of differences between the three groups of *T. brucei* strains studied by us and the one studied by Stuart [40] is given in Table IV. The genetic diversity in *T. brucei* maxi-circles appears to be similar to that of the mtDNAs of the rodents studied. The differences in the case of *T. brucei* may actually be somewhat exaggerated because one of the four polymorphic sites lies within the variable region and the other at its border. As long as this region and the cause of its variability have not been defined more precisely, we have included these sites in the comparison.

Our results extend the previous observation [20] on extensive sequence differences between the mini-circles of strains 427-60 and EATRO 1125 on two points. First, the analyses with *Taq*I show that sequence divergence is a general feature of *T. brucei* strains and not an incidental finding of the two strains analysed before. Second, our results suggest that sequence evolution is still slow enough to allow the use of mini-circle fragment distribution as a sensitive tag to identify different strains. In fact, our only evidence that strains that fall in the same group on the basis of maxi-circle sequence analysis (e.g. 31 and 839, and 127 and 1125) are not identical, comes from this analysis of mini-circle fragments and an analogous analysis of nuclear DNA fragments reported elsewhere [51].

Finally, we would like to stress the point — briefly mentioned already in Materials and Methods — that we have recently found that the strains in groups 2

TABLE IV
ESTIMATED GENETIC DIFFERENCES BETWEEN GROUPS OF *TRYPANOSOMA BRUCEI* STRAINS, DEDUCED FROM RESTRICTION SITE POLYMORPHISMS

The values above the diagonal are the genetic differences in base substitutions per nucleotide, the values under the diagonal are the number of sites analysed (4 base-pair enzymes/6 base-pair enzymes). For the calculation of the average base substitution per nucleotide we assume that the loss or gain of restriction sites is due to a single base-pair change and, in view of the high homology between the maxi-circles analysed here, this seems reasonable. The sequence difference between two groups expressed as average base substitutions per nucleotide is calculated as the sum of the sites that differ between those two groups (see Table III) divided by the total number of nucleotides in all sites screened in both groups (see Table II). For the calculations we have used strain 31 as representative of group 2 and strain 127 for group 3. Note that the total number screened may be lower than that inferred from Table II, because a site absent in two strains is not counted as screened if these two are compared.

	Average base substitutions per nucleotide			
	Group 1	Group 2	Group 3	Group 4
Group 1 (427)	0	0.022	0.022	0.014
Group 2 (31, 839)	20/9	0	<0.008	0.021
Group 3 (127, 1125)	21/9	18/8	0	0.021
Group 4 (164)	2/11	0/8	0/8	0

and 3 are closely related to 'rhodesiense' variants of *T. brucei* which are infective to man. In addition, another 'rhodesiense' variant is similar to the 427 strain (Borst, P., Fase-Fowler, F. and Gibson, W.C., unpublished results). This confirms that the genetic differences between *T. brucei brucei* and *T. brucei rhodesiense* are very small (see Ref. 26). It may, therefore, be prudent to consider every *T. brucei* strain potentially infective to man.

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