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Sequence heterogeneity and anomalous electrophoretic mobility of kinetoplast minicircle DNA from *Leishmania tarentolae*

(Recombinant DNA; plasmid pBR322; M13 phage vectors; molecular cloning in *E. coli*)

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SUMMARY

Several unit-length minicircles from the kinetoplast DNA of *Leishmania tarentolae* were cloned into pBR322 and into M13 phage vectors. The complete nucleotide sequences of three different partially homologous minicircles were obtained. The molecules contained a region of approx. 80% sequence homology extending for 160–270 bp and a region unique to each minicircle. A 14-mer was found to be conserved in all kinetoplast minicircle sequences reported to date. The frequency distributions of various minicircle sequence classes in *L. tarentolae* were obtained by quantitative gel electrophoresis and by examination of the "T ladder" patterns of minicircles randomly cloned into M13 at several sites. By these methods we could assign approx. 50% of the total minicircle DNA into a minimum of five sequence classes. A sequence-dependent polyacrylamide gel migration abnormality was observed with several minicircle fragments both cloned and uncloned. The abnormality was dependent on the presence of a portion of the conserved region of the minicircle.

INTRODUCTION

The kDNA of the kinetoplastid protozoa contains two species of molecules: the maxicircles, which contain the mitochondrial ribosomal and structural genes, and the minicircles, whose function is unknown. There are approx. 10^4 minicircles within a kDNA network. In general, minicircle DNA from the several species that have been studied (reviewed by England et al., 1982) has the following general characteristics: (1) The circles are catenated together to form the network structure, although there is a small percentage of unattached circles that have been shown in the case of *Crithidia* to be replicative intermediates (Englund, 1979). (2) Within any one species, the circles are fairly uniform in size, but among species the size varies from approx. 900 bp in *Leishmania* to 2300 bp in *Crithidia*. (3) There is sequence heterogeneity among the minicircles from any one clonal population, which is limited to a variable region. (4) Sequence changes among the minicircle population within any one species occur rapidly in nature. (5) No sequence homology exists between the minicircles and the maxicircle DNA of a given species.

The sequences of two cloned minicircles from *Trypanosoma brucei* (Chen and Donelson, 1980), and one minicircle from a *T. equiperdum* strain (Barrois et al., 1982) have been reported. In addition a partial

Abbreviations: bp, base pairs; EtBr, ethidium bromide; kDNA, kinetoplast DNA; ORF, open reading frame; TBE buffer, 89 mM Tris · HCl (pH 8.3), 89 mM boric acid, 2.5 mM EDTA; T ladder, the T lane of a dideoxy chain termination sequencing gel, as described in MATERIALS AND METHODS, section e and in RESULTS, section e.

sequence of a cloned minicircle from *L. tarentolae* and a partial sequence of a cloned minicircle from *T. cruzi* have been published (Barker et al., 1982; Van Heuverswyn et al., 1982). The two *T. brucei* and the *T. equiperdum* minicircles were found to share a short (120–130 bp) region of sequence homology and to possess potential open reading frames of no more than 51, 72 and 22 amino acids, respectively. However, no minicircle transcription has been reported.

In the present report we describe the cloning and sequencing of several minicircles from *L. tarentolae* and the distribution of several of the minicircle sequence classes. We also describe anomalous electrophoretic migration of minicircle DNA in acrylamide, which seems to be dependent on the presence of the conserved region of the minicircle and, in one case, on an adjacent *Eco*RI site.

MATERIALS AND METHODS

(a) Cells

Escherichia coli RR1 cells were used for transformation by the pBR322 plasmids. *E. coli* strains JM101 and JM103 were used as hosts for transfection and transformation, respectively, with the M13mp8 and mp9 phages (Messing and Vieira, 1982). The *L. tarentolae* cells (clonal strain C-1) were grown as described previously (Simpson, 1979).

(b) DNA isolation

Networks of kDNA were isolated from stationary phase *L. tarentolae* as described (Simpson, 1979). For the experiment in Fig. 6, kDNA was sonicated and centrifuged in CsCl-EtBr. The lower band was recovered and sedimented in a 5–20% sucrose gradient to separate closed monomeric minicircles from dimers and trimers. The upper CsCl band was also sedimented in sucrose to separate the unit-length linear band from open monomeric and dimeric circles (Simpson and da Silva, 1971). The closed monomeric minicircle DNA was nicked by the DNase-EtBr method of Greenfield et al. (1975) to produce open monomeric minicircles.

The pLt19, pLt26 and pLt154 plasmid DNAs were isolated by CsCl-EtBr centrifugation as described previously (Simpson et al., 1979).

(c) Isolation of DNA from agarose

DNA was eluted from agarose by electroelution inside dialysis bags (McDonell et al., 1977) or by binding to DEAE-nitrocellulose membranes (Schleicher and Schuell NA-45).

(d) Construction of recombinant plasmids and phage

The pBR322-derived recombinant plasmids, pLt19, pLt26 and pLt154, were selected by hybridization of probes to colonies containing plasmid clones obtained by ligation of digested total kDNA with digested plasmid DNA.

Colony hybridization was performed as described previously (Grunstein and Hogness, 1975). Labeling of DNA probes by nick translation was performed as described (Rigby et al., 1977).

(e) DNA sequencing

Sequencing was performed either by the Maxam and Gilbert (1980) method or by the dideoxy chain termination method of Sanger et al. (1977).

RESULTS

(a) Cloning of minicircles

kDNA from *L. tarentolae* was digested with *Bam*HI or *Hin*dIII and ligated with digested pBR322 DNA. One *Bam*HI, ampicillin-resistant, tetracycline-sensitive clone (pLt19) was selected by colony hybridization using total kDNA as a probe. Two additional minicircle clones (pLt26 and pLt154), with inserts at the *Hin*dIII site, were also selected. Colony hybridization of the minicircle clones showed that all three shared some sequence homology (not shown), but they did not show any detectable homology with the cloned 6.6-kb maxicircle fragment, pLt120, or with a total maxicircle probe, nor with cloned minicircle or maxicircle DNA from *T. brucei* (Simpson and Simpson, 1980). Digestion of the chimeric plasmids released inserts of approx. 870 bp (Fig. 1), with the pLt19 insert running somewhat more slowly on the gel than the pLt26 and pLt154 inserts. The pLt19 plasmid was shown by digestion with a single cutting enzyme to contain a double

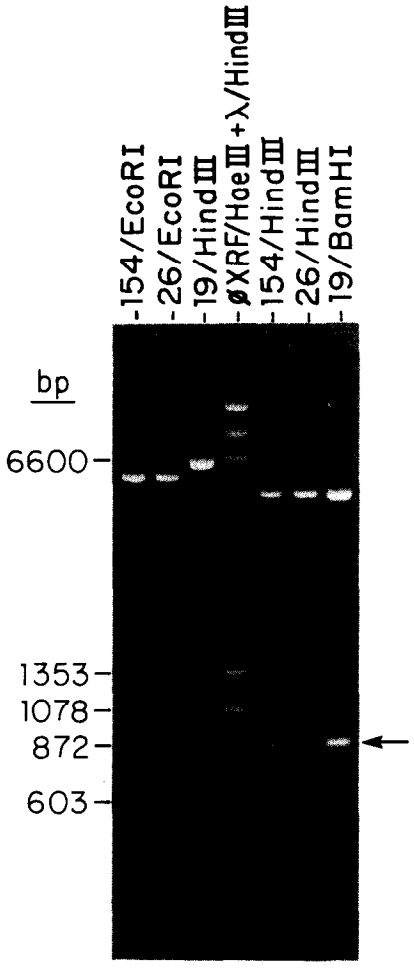

Fig. 1. Gel electrophoresis of minicircle plasmids digested with several enzymes (1% agarose in TBE buffer). Unit-length minicircle inserts (arrow) are released in the 19/*Bam*HI, 26/*Hin*dIII and 154/*Hin*dIII digestions. The pLt19 plasmid has a single site for *Hin*dIII and the pLt26 and 154 plasmids have single sites for *Eco*RI (see RESULTS, section **a**). ϕXRF, RF form of ϕX174 DNA; slash indicates digestion by specified enzyme.

insert, whereas pLt26 and pLt154 contained single inserts (Fig. 1). The double insert in pLt19 was shown by restriction-site mapping and by Maxam–Gilbert sequence analysis to consist of two identical unit length minicircles joined in the head-to-tail orientation (not shown). All three minicircle inserts were subcloned in both orientations in M13mp8 and mp9 (mLt19A, mLt19B, mLt26A, mLt26B, mLt154A, mLt154B). In addition, an *Eco*RI unit-length minicircle that had been previously cloned into the yeast shuttle vector YIp5 (pKSR1) (Kidane, G.Z. and Simpson, L., unpublished results) was subcloned into M13mp8 and mp9 (mKSR1A, mKSR1B) in both orientations.

(b) Sequence analysis of cloned minicircles

Restriction maps of the minicircle inserts in pLt19, pLt26 and pLt154 were derived by double and triple digestions (Fig. 2). The construction of the restriction maps was complicated by the fact that several minicircle fragments exhibited an abnormal electrophoretic mobility in acrylamide gels. The M_rs derived from electrophoresis in agarose were therefore used for the map constructions.

Partial sequences were obtained by Maxam–Gilbert analysis of end-labeled restriction fragments of the minicircle inserts in pBR322. Complete sequences (Fig. 3A–C) were obtained by dideoxy chain termination analysis of the minicircles cloned in M13. The insert sizes were 874 bp for pLt19 and 824 bp for pLt26. The sequence of the *Eco*RI insert in the M13 clone mKSR1 proved to be identical to the *Bam*HI insert in pLt19 (not shown), indicating that this represents the entire minicircle sequence. In

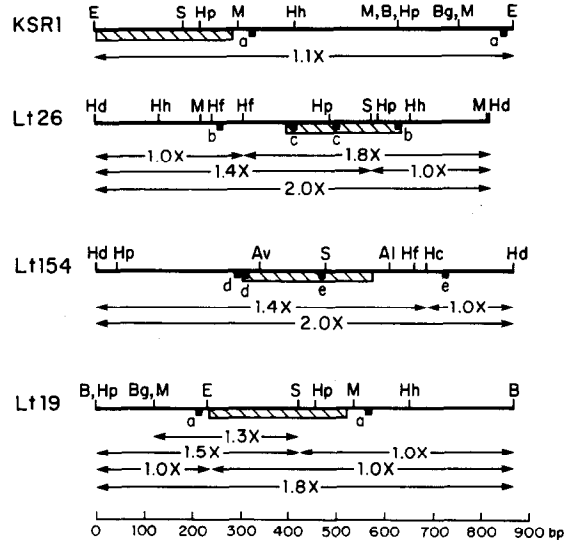

Fig. 2. Restriction maps of the cloned minicircle inserts from the pLt19, pLt26 and pLt154 plasmids. The homologous regions are indicated by hatched boxes and the fragments that migrate anomalously in acrylamide are indicated by the "abnormal migration coefficients" (the ratio of the apparent M_rs from migration in 5% polyacrylamide vs. migration in 1.5% agarose) given below the fragments (e.g. 1.1X or 2.0X). Internal repeats of 10 bp or greater (determined from the sequences in Fig. 3) are indicated by the small black boxes labeled a–e. Abbreviations: E, *Eco*RI; B, *Bam*HI, Hd, *Hin*dIII; Bg, *Bgl*II; M, *Mbo*I; S, *Sma*I; Hp, *Hpa*II; Hc, *Hin*cII; Hf, *Hin*fI; Al, *Alu*I; H, *Hae*III; Hh, *Hha*I.

A. Sequence of pKSRI minicircle DNA

```
        10          20          30          40          50          60
CCAAAAATGT CAAAAAATAG GCAAAAAATG CCAAAAATCC CAAACTTTTT AGGTCCCTCA

        70          80          90         100         110         120
GGTAGGGGCG TTCTCCGAAA ACCGAAAAAT GCATGCAGAA ACCCCGTTCA AAAATCGGCC

       130         140         150         160         170         180
AAAATCGCCA TTTTTTCAAT TTTCGTGTGA AACTAGGGGT TGGTGTAAAA TAGGGGTGGG

       190         200         210         220         230         240
GCTCCCCGGG GTAATTCTGG AAATTCGGGC CCTCAGGCTA GACCGGTCAA AATTAGGCCT
   SmaI                                                HpaII
       250         260         270         280         290         300
CCTGACCCGT ATATTTTTGG ATTTCTAAAT TTTGTGGCTT TAGATGTGGG AGATTTGGAT

       310         320         330         340         350         360
CTAACACGTG AGGATAAGGG TCTGCATGGG CTTGTGTAGG CGTGTAGACT GGCATCCAAG
                             a
       370         380         390         400         410         420
GGCCCCTACC AAAGATGGAT TTGATATTTA AGAGTTTATG TGCTTGGACT TTGTTAGGCG

       430         440         450         460         470         480
CATGCGATAC AGCGGTGGTG TGGCTGTGTT GGACAACGTG ACACACTCGG GCGATAGCCA

       490         500         510         520         530         540
GCATGGATAG CTAACGTGGA TACGTTGAGG CTGGGGTATG CCTCTTAAGC CCTTATAACT

       550         560         570         580         590         600
TATATTACCA TATATAGTAA ATAGGGGCAA TGTCGATAAG ACACGTATAG CCTAGGTGGT

       610         620         630         640         650         660
AACAGCTAGG ATATGCGGTG ACATAGTATA GGATCCGGTA GCCTACATGG GTAACCCGTT
                                   BamHI
       670         680         690         700         710         720
GGAGAGGAGT CGACAGTAGG GTAGGATTCT ATCTTTAATT TACAGTAGAG ATATATATTT

       730         740         750         760         770         780
TTCACTTCAA CCACATTGGC GTTTTGCTGG ATGTTATTTA AGATCTAGAC TAGACGCTAT
                                            BglII
       790         800         810         820         830         840
CGATAAAGTT TAAACAGTAC AACTATCGTG CTACTCACCT GTTGCCAAAC ATTGCAAAAA

       850         860         870
TGCAAAATTG GGCTTGTGGA CGCGGAGAGA ATTC
           a                EcoRI
```

B. Sequence of pLt26 minicircle DNA

```
        10          20          30          40          50          60
AAGCTTATCG GTGTTAGGTG AGTGCGTTGA TGACTATCGA CGTTCGTTAG CCTTGGATAA
HindIII
        70          80          90         100         110         120
CGTTGAGCAT TAGGCATTTG GTAATAGGAG GCTATAATGA GCAATTTATA ACTTATATTA

       130         140         150         160         170         180
CCATTGACTT TGTGGCGCAT TATAGGAGCC TAGGAGGCGA CGATTAGACC TATAAGATTA
              HhaI
       190         200         210         220         230         240
CACGAGTTGT ATACGATGTA TTTGAATAGT GTGCGTTATA TTTAGATCAT GACTTAGATA

       250         260         270         280         290         300
TACGACTCTA CATGTTCCCT ATATTTATAC CGAGTTTAAT GTTTAACGAG GCCTCCTAGA
  HinfI          b
       310         320         330         340         350         360
CGTTTATGAA TCCATCTACG GAGAAGTCCT TTACACTTCC ATACTGTGTT TCCGCTACTG
      HinfI
       370         380         390         400         410         420
AGCACTGGTC TATAAGCCAG ATGCATTTTC AGAAAATTCT AAATGCCAAA ATGCCAAAAT
                                                  c           c
       430         440         450         460         470         480
AGCCCAAAAT CCCAAACTTT TAGGTCCCTA GGTAGGGGCT CTCCGAAACC GAAAAATCAT

       490         500         510         520         530         540
GCAGAAACCC GGTTCAAAAA AATGCCAAAA AATCGTCCAT TTTTTACGAT TTTTCTATAA
     HpaII          c
       550         560         570         580         590         600
AACTTAGGGG TTGGTGTAAA AATAGGGGTG GGGCTCCCCG GGGATTTCCG GGCCCCTCAG
                                    SmaI
       610         620         630         640         650         660
GTTTCGACCC TCAAAAATAA GGCCTTTGGA CCCTATATTT TGCACGTGGC TAAATTTAGG
                                      b
       670         680         690         700         710         720
CGCATTAGAA TCGTGGCTTT TTCCATCTAA GGCATAGGAT TAGGCCTGTG TAATGCTGGT

       730         740         750         760         770         780
GTTTCGAGAT GTCGGGATAG TGACAGGTAC CCCTACCTAG CTGGCTAGCA TGTGGTTTGT

       790         800         810         820
GGTAAGCGTG TGGCGGCATG GGGTTAATGA TGCGAGATGA TGATCG
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Fig. 3. The nucleotide sequences of the three cloned minicircles, (A) pKSR1; (B) pLt26; and (C) pLt154. The pLt19 sequence is identical to the pKSR1 sequence but is linearized at the *Bam*HI site. The 14-mer indicated by the box is a sequence that is conserved in all kDNA minicircle sequences reported to date, including the *T. brucei*, *T. equiperdum* and *T. cruzi* minicircles (12 of the 14 nucleotides are conserved in the *T. cruzi* minicircle).

C. Sequence of pLt154 minicircle DNA

```
        10          20          30          40          50          60
TAGCTGATCT AGGTAACTAG TTCAGGATTT GGACAACATG TATCCGGCTT GTAAAGTGAA
                                                HpaII
        70          80          90         100         110         120
CATTGTGTTA TATCAATTTT AACTAACGTA ATCTATATTT AACACTCTAT GATTTCTCTA

       130         140         150         160         170         180
TGTTTTTTGT TGTGTTTGTA TATTAAGTTA ATGTATGCAG TAGAATAGAG ACAGTATACA

       190         200         210         220         230         240
ACTATCGCCT ACTACTGTGT TCGTCAAGCG AAAAAAGTAA TTCGGCCGAA GTTGGTGCAT

       250         260         270         280         290         300
TGGTTTGCTG TATGCGCATT AAATTTGGGA TAAGAAAACT TTAAAAAATT TTGCCAAAAT
                                                              d
       310         320         330         340         350         360
TTTTTGCCAA AAAATGCCCA AAAATTCCCA AACTTTTTTA GGTCCCTCAG GTAGGGGCGT
  d                                                  AvaII
       370         380         390         400         410         420
TTCTCCGAAA CCGAAAAATG CATTGCACGA AACCCCCTTC AAAATTCGGC CAAAATTCGC

       430         440         450         460         470         480
CATTTTTTCG AATTTTCGTG TGAAACTAGG GGTTGGTGTA AAATAGGGGT GGCGCACCCC
                                                        e       SmaI
       490         500         510         520         530         540
GGGCCCTCCC ACGGAAATTC GGGGATTTAT TTAGGCGGGT GAAATAAGGC CTCCTACCCC

       550         560         570         580         590         600
GTATATTTGT ATGCTAAATT TTCTCGCTTA GAATGTGGCG AATTTCCATC AGAACGCTGG

       610         620         630         640         650         660
ATACACGCTA TGCAGCTTGC ATGGTGTTTG GATAAGTGGC TTTGTAAGCC TGTGTGACAC
             AluI
       670         680         690         700         710         720
ATGTGACTCT TCGAATGTTG TGGTGTTGAC CGCATTGAGT TGATCGATAT GGAGATAGGC
                            HincII
       730         740         750         760         770         780
TACGGGTGGC GCATGTTGTA ATGCGTCATG ATGTTAGGGG TTAGGTAAAC GTTGGCATCG
  e
       790         800         810         820         830         840
GTTACGCATG GCATACGGTA TACGTGAGAC CCATAATATA TGATAATATA AGTTATATTA

       850         860         870
TCATTTAAGC CGATCGACGT TAGGCGAAGC TT
                          HindIII
```

The two palindromes in the pLt154 sequence are indicated by wavy underlines. The underlined sequences, a–e, are internal repeats of ten or more nucleotides (see Fig. 2).

the cases of the inserts in pLt26 and pLt154, overlapping unit length clones were not obtained by using different enzymes, so that we cannot be sure that these represent entire minicircle sequences. However, the apparent sizes of the linearized minicircle main bands released from network DNA by digestion with *Eco*RI and *Hin*dIII (Fig. 7) are consistent with there being a *Hin*dIII fragment class slightly smaller than the *Eco*RI class. The complete nucleotide sequences are shown in Fig. 3A–C. Comparison of the sequences shows a conserved region of approx. 200 bp in all three fragments as indicated on the restriction maps in Fig. 3 and presented in the alignments in Fig. 4. The homologies although not perfect are clearly significant. Several small internal perfect repeats of 10 bp or greater are present in all three fragments as indicated on the restriction maps in Fig. 2. Perfect dyad symmetries of 11 and 12 bp are present in the pLt154 sequence (indicated in Fig. 3C).

Comparison of the *L. tarentolae* sequences with the published *T. brucei* (Chen and Donelson, 1980) and *T. equiperdum* (Barrois et al., 1982) sequences

Fig. 4. Alignment of the conserved regions of the (A) KSR1 and Lt154 sequences, (B) KSR1 and the Lt26 sequences, and (C) Lt26 and the Lt154 sequences. The non-homologous sites are indicated by asterisks. The degree of homology (% matching bases) is 83% for the KSR1-Lt154 alignment, 77% for the KSR1-Lt26 alignment and 81% for the Lt26-Lt154 alignment. The numbering of the nucleotides refers to the sequences in Fig. 3A–C.

shows little sequence homology. There is, however, a 14-bp sequence (5′-TAGGGGTTGGTGTA-3′) that is present in the conserved regions of the three *L. tarentolae* fragments (indicated by boxes in sequences in Fig. 3) and also present in the conserved regions of the *T. brucei*, *T. equiperdum* and *T. cruzi* (12-mer in *T. cruzi*) minicircles. Comparison of the three *L. tarentolae* minicircle sequences with the partial sequence of a cloned *Eco*RI-linearized *L. tarentolae* kinetoplast DNA minicircle reported by Barker et al. (1982) shows that the KSR1 minicircle has a sequence identity of over 95%, implying that members of the same minicircle class were cloned in both cases. All of the differences involve single nucleotides, and it is not known if these represent true polymorphisms or sequencing errors.

The distribution of termination and initiation codons in all six reading frames for the three *L. tarentolae* minicircle sequences is shown in Fig. 5. It is interesting that, as in the *T. brucei* minicircle sequences (Chen and Donelson, 1980), there are short ORFs in or near the conserved regions; these are not, however, always the longest ORFs. The presumptive translated proteins show no similarity in amino acid sequence with those from the ORFs in the *T. brucei* minicircles.

(c) Anomalous electrophoretic migration of minicircle fragments

We reported previously (Simpson, 1979; Simpson et al., 1980) that linearized minicircle DNA isolated from kDNA ran more slowly in acrylamide gels than predicted from the mobility in agarose. This effect was also observed with unit-length minicircle inserts and minicircle fragments isolated from recombinant plasmids. This can be seen in the diagrams of Fig. 2, in which the relative increase in apparent M_r calculated from electrophoresis in 5% polyacrylamide vs. 1.5% agarose (the "abnormality coefficient") is given beneath each fragment tested. It is clear that the *Eco*RI site in the Lt19-KSR1 minicircle insert is important for this phenomenon since the Lt19 insert has an abnormality coefficient of 1.8×, whereas the KSR1 insert has a coefficient of only 1.1×. Also, digestion of the Lt19 insert at the *Eco*RI site produces two fragments, both of which run normally. However, digestion of the Lt19 insert at the *Sma*I site or the *Sma*I and *Bgl*II sites yields fragments that run abnormally. Similar results were obtained with the Lt26 and Lt154 inserts. These data imply that the presence of at least a portion of the conserved region is essential for the fragment to exhibit this abnormal migration behavior, with the extent of the abnormality being roughly dependent on the amount of the conserved region present. The terminal portion of the conserved region and the *Eco*RI site in Lt19 seems to be particularly important in this phenomenon.

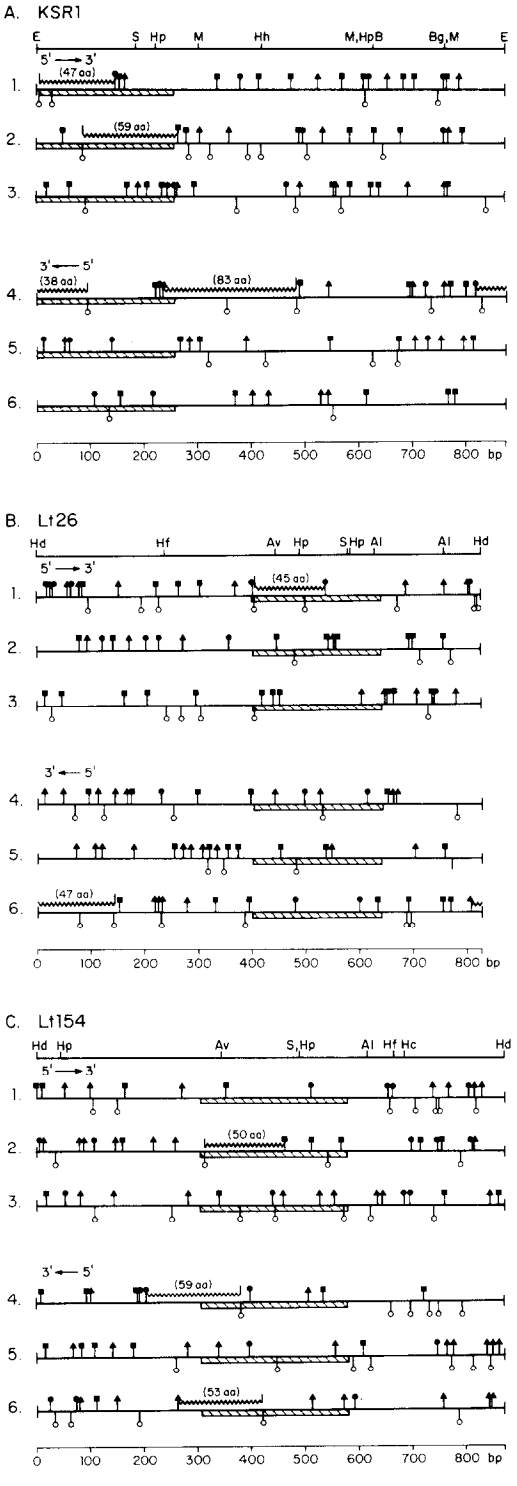

Fig. 5. Distribution of termination and initiation codons (universal genetic code) in all six reading frames of the three (A, B, C) minicircle sequences (see Fig. 3). Several open reading frames with the indicated number of amino acids are indicated by the jagged lines. The hatched boxes represent the conserved regions. Symbols: initiation codon ATG = ♁; termination codons TAA = ↑, TGA = ⚲, and TAG = ■.

(d) Heterogeneity of intact minicircles

Closed monomeric minicircle DNA was isolated from mildly sonicated network DNA by sucrose gradient centrifugation. As shown in Fig. 6, the closed minicircles exhibit a heterogeneous electrophoretic banding pattern in acrylamide-agarose. The nicked monomeric minicircle DNA liberated by sonication or produced by single-nicking closed monomeric minicircles gives a three-band pattern, with one major band and two minor bands (x, y, z in 6B). From the above sequences of the KSR1, Lt26 and Lt154 minicircles, it is clear that size differences

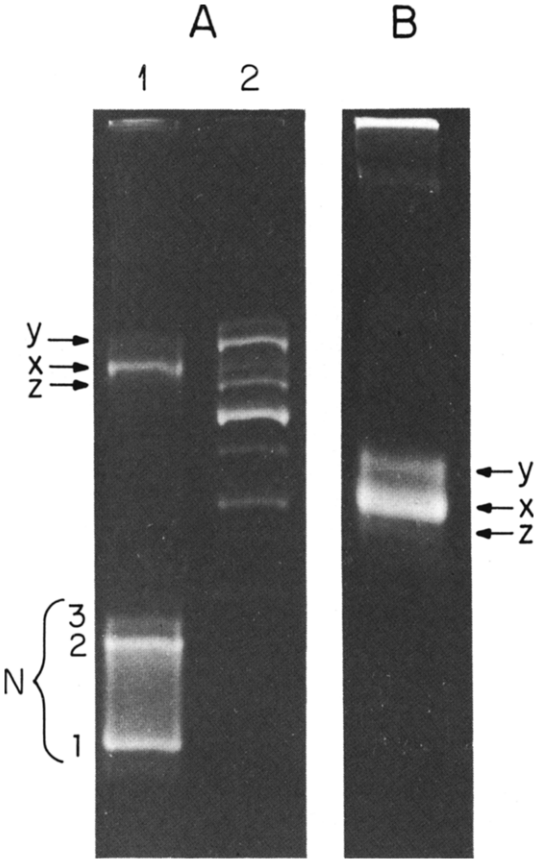

Fig. 6. Gel electrophoresis of minicircles and linearized minicircles from kinetoplast DNA (1.5% polyacrylamide–0.5% agarose in Tris acetate-EDTA buffer). (A) Lane 1, Nicked minicircles and unit-length linears, isolated as described in MATERIALS AND METHODS, section **b**. Bands x, y and z contain nicked minicircles (seen better in lane B). Bands N-1, 2 and 3 contain linearized minicircle DNA. Lane A2, closed monomeric minicircles from sonicated kDNA, isolated as described in MATERIALS AND METHODS, section **b**. (B) Open monomeric minicircles obtained by digesting closed monomeric minicircles with DNase I in the presence of EtBr.

probably do exist between different minicircle sequence classes. These size differences probably account for the presence of three bands of open minicircle DNA and also for the non-Gaussian banding pattern of the superhelical minicircles in Fig. 6A (lane 2). The three bands and the smear labeled "N" in the sonicated kDNA lane in Fig. 6A (lane 1) most likely consist of linearized unit-length minicircles that exhibit different degrees of the abnormal electrophoretic migration behavior. We showed previously (Simpson, 1979) that the N1 band comigrated in acrylamide with the major *Eco*RI-linearized minicircle band in a digest of total kDNA and that the N2 and N3 bands comigrated with the retarded minor *Eco*RI minicircle band. Our interpretation is that cleavage of a minicircle at a site outside the conserved region yields a linear molecule that shows the maximum migration abnormality and that cleavage within the conserved region, particularly near the 5′ terminal portion, yields a linear molecule with little migration abnormality. The existence of two discrete abnormally migrating bands, N2 and N3, may be due to size differences of different minicircle sequence classes.

(e) Distribution of minicircle sequence classes

Determination by gel electrophoresis of the relative abundance of minicircles possessing a single *Eco*RI, *Bam*HI or *Hin*dIII site is complicated by the fact that a substantial number of minicircles lack these sites, and therefore these enzymes do not completely disrupt the network; undigested network DNA does not enter the gel and can suffer a variable amount of loss by washing out at the origin, leading to erroneous results in densitometric analysis of stained gels. However, enzymes such as *Msp*I which have sites within the conserved region of the minicircles completely disrupt the network structure as shown in Fig. 7, and the amount of released minicircles and minicircle fragments can be monitored easily by densitometric analysis of stained gels.

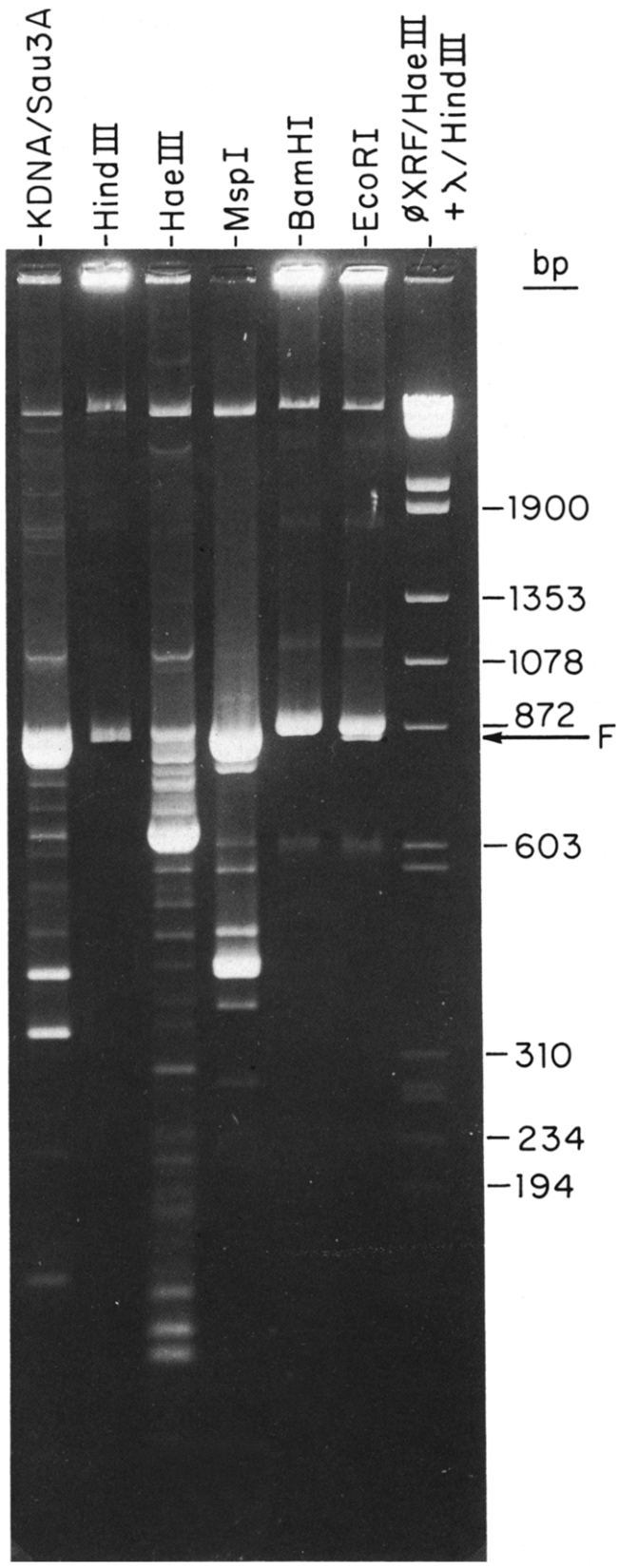

Fig. 7. Gel electrophoresis of kDNA digested with several enzymes (1% agarose in TBE buffer). The enzymes used are indicated above each lane. Reference fragments were DNA digested with *Hin*dIII and ϕX174 RF DNA digested with *Hae*III. Band F is discussed in RESULTS, section e.

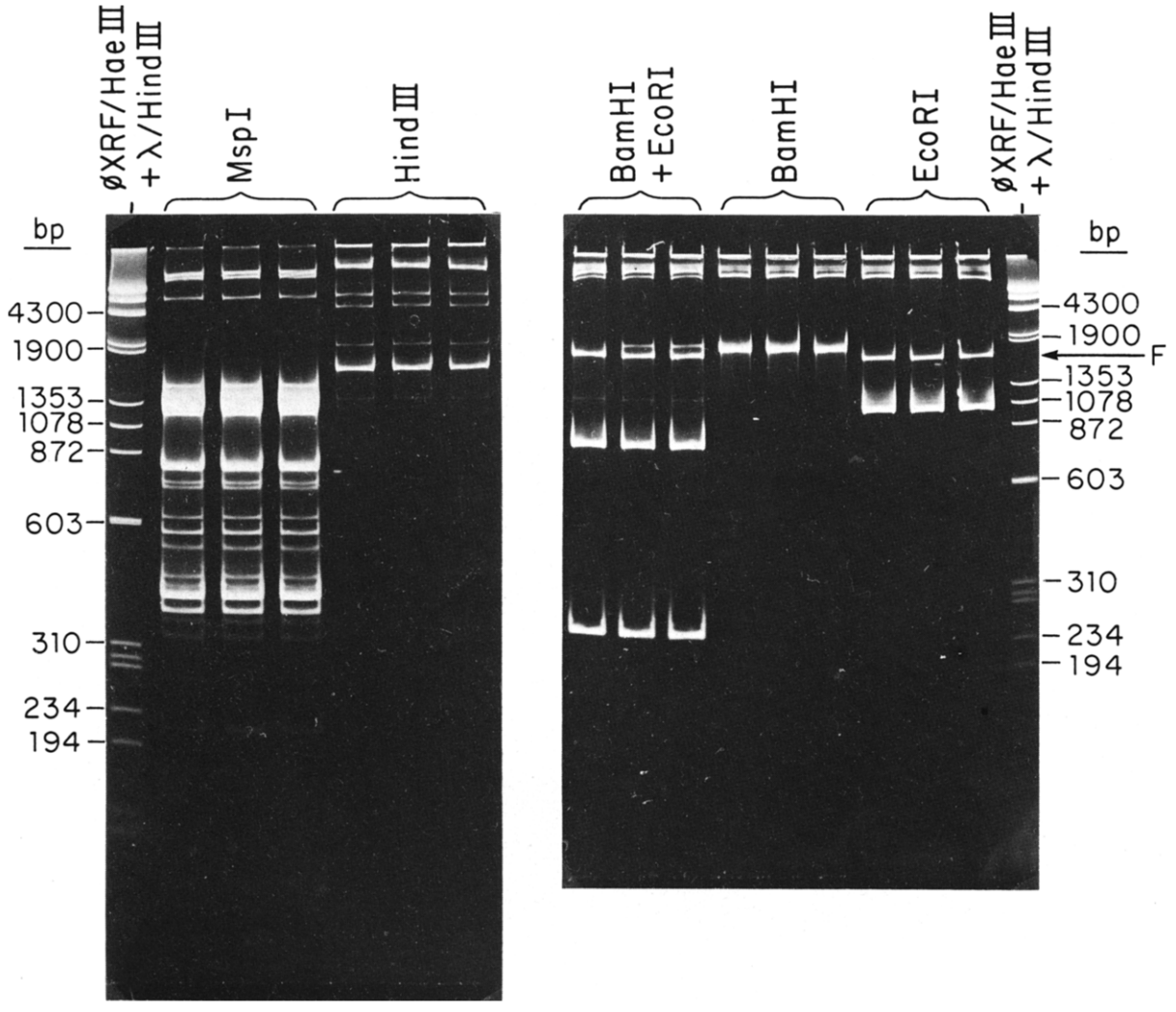

Fig. 8. Polyacrylamide gel (5%) electrophoresis in TBE buffer of digested kDNA. Equal amounts of kDNA were digested with excess *Eco*RI, *Bam*HI, *Eco*RI + *Bam*HI, *Hin*dIII and *Msp*I, and the digests were loaded into three wells each, as indicated. Densitometer tracings of the negative were used to quantitate the relative amounts of the individual bands as described in Fig. 10. For reference fragments see Fig. 7.

Note in the agarose gel in Fig. 7 the presence of a minor band (arrow labeled "F") migrating slightly ahead of the major unit-length *Eco*RI minicircle band and also the presence of a second minor *Eco*RI band migrating with the 603-bp ϕX174 RF band. In the acrylamide gel in Fig. 8, the minor unit-length *Eco*RI band (arrow labeled "F") migrates more slowly than expected from its mobility in agarose (see Fig. 7), as do the unit length *Bam*HI and *Hin*dIII minicircle bands, whereas the major *Eco*RI band runs almost normally. The presence of a single *Bam*HI site in the major *Eco*RI minicircle band DNA and the absence of a *Bam*HI site in the minor *Eco*RI minicircle DNA (band labeled "F") is shown by the double *Eco*RI + *Bam*HI digestion in Fig. 8.

The unit-length *Bam*HI minicircle band was shown to be reasonably homogeneous by redigestion of a gel-isolated band with *Hae*III or *Sau*3A (results not shown). Likewise, the major unit-length *Eco*RI minicircle band was eluted from a polyacrylamide gel and shown by redigestion with *Msp*I or *Hae*III to be reasonably homogeneous (results not shown). This band represents the KSR1 minicircle sequence class. Isolation from acrylamide and redigestion of the mi-

nor unit-length *Eco*RI minicircle band with *Msp*I and *Hae*III gave inconclusive results; the DNA appeared somewhat heterogeneous but this may have been due to incomplete digestion and the presence of partial digestion products.

To measure the relative abundances of minicircles possessing a single *Eco*RI, *Bam*HI or *Hin*dIII site, equal amounts of kDNA were digested with *Msp*I, *Eco*RI, *Bam*HI and *Hin*dIII, electrophoresed in agarose, and the relative amounts of each band determined densitometrically (Fig. 9, Table I). Approx. 41% of the minicircles contained a single *Eco*RI site. Approx. 20% of the minicircles contained a single *Bam*HI site and 10% a single *Hin*dIII site. The minor *Eco*RI band that is selectively retarded in polyacrylamide and lacks a *Bam*HI site represents 36% of the total unit-length *Eco*RI minicircle DNA. Therefore, the major class of *Eco*RI-cleavable minicircles with single *Bam*HI sites (= KSR1 class) represents approx. 26% of the total minicircle DNA in the network. The difference between this value and the value of 20% derived from agarose gel electrophoresis of *Bam*HI-digested kDNA is attributed to experimental error. Since it was shown by double digestion that both of the unit-length *Eco*RI minicircle bands lack a *Hin*dIII site, it can be concluded that the total minicircle DNA released by *Eco*RI, *Bam*HI and *Hin*dIII represents approx. 50% of the total minicircle DNA.

To obtain a more detailed estimate of the number and type of minicircle sequence classes, total kDNA was digested with several enzymes, ligated with M13mp8 and mp7 RF DNA, and used to transform *E. coli*. Clear plaques were selected and tested for unit-length minicircle inserts by direct gel electrophoresis. These minicircle inserts by direct gel electrophoresis. These minicircle clones were screened for strand orientation by hybridization against one test clone. Dideoxy chain termination reactions with ddTTP ("T" ladders) were then run using a single-stranded M13 primer and the resulting patterns compared. As shown by the results in Table II and by the diagram in Fig. 10, the A–C and D–E patterns corresponded to the KSR1 sequence, the H–L patterns to the Lt26 sequence, and the B–G patterns to the Lt154 sequence. The only new patterns seen that could not be interpreted as rearrangements or deletions of known patterns were the F and P patterns, which represent new sequence classes. The F class

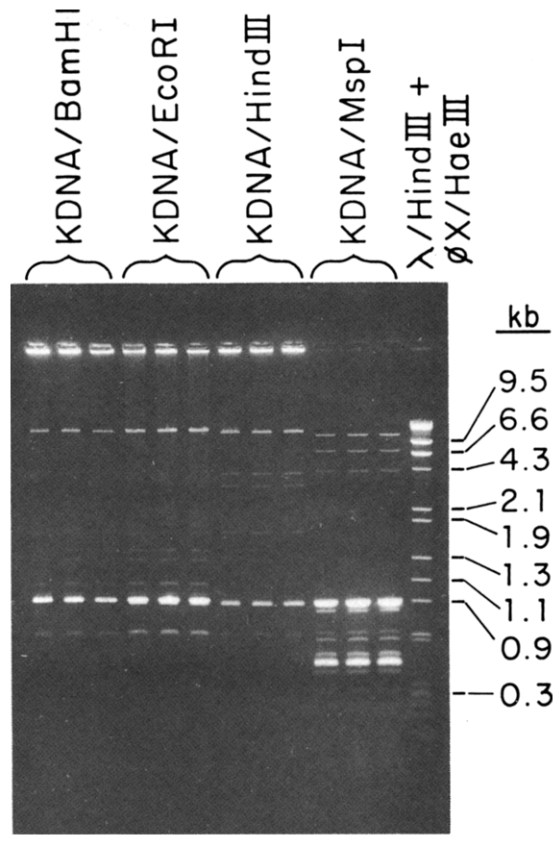

Fig. 9. Agarose gel electrophoresis of restriction digests of kDNA. (1% agarose in TBE buffer). Equal amounts of kDNA digests with *Msp*I, *Hin*dIII, *Eco*RI and *Bam*HI were loaded into three lanes each, as indicated. Densitometer tracings of the film were used to quantitate the relative amounts of the individual minicircle fragment bands. Areas under peaks were measured using a digitizer tablet and a computer program that resolves Gaussian peaks. Since all minicircles in the network appear to contain at least one *Msp*I site, the *Msp*I minicircle bands were summated and used as the 100% minicircle DNA value, and the relative amounts of the *Eco*RI, *Bam*HI and *Hin*dIII bands were calculated. Note the single unit-length maxicircle bands in the *Eco*RI and *Bam*HI lanes, and the several high molecular weight maxicircle fragment minor bands in the *Hin*dIII and *Msp*I lanes. In addition, faint ladders of minicircle catenanes can be seen in the *Eco*RI, *Bam*HI and *Hin*dIII lanes. Note the presence of undigested kDNA at the origin in the *Eco*RI, *Bam*HI and *Hin*dIII lanes and the absence of kDNA at the origin in the *Msp*I lane.

was identified by blot hybridization (results not shown) as the minor minicircle band arising from *Eco*RI digestion (arrow in Fig. 8) that exhibited anomalous migration in acrylamide. We conclude that there are at least five different minicircle sequence classes, which represent approx. 50% of the total minicircle DNA.

TABLE I

Percentage of total minicircles possessing single sites for specified enzymes

	Enzyme	Percentage [c]	
		Exp. 1	Exp. 2
(A) Agarose [a]	*Hin*dIII	9.2	10.0
	*Eco*RI	37.4	45.8
	*Bam*HI	21.4	20.3
(B) Polyacrylamide [b]	*Eco*RI	Band (1)	74
		Band (2)	26 [d]

[a] Equal amounts of digested kDNA were electrophoresed in 1% agarose. The gel was photographed and the relative peak areas as measured by densitometry were compared to the *Msp*I minicircle bands, which were summated and used as the 100% value.
[b] *Eco*RI-digested kDNA was electrophoresed in 5% polyacrylamide. The gel was photographed and the relative peak areas of the two unit-length minicircle bands were measured.
[c] This represents the percent of total minicircle DNA for the unit length minicircle bands produced by digestion with the specified enzymes, calculated as described in a above.
[d] Band (2) was retarded in polyacrylamide relative to Band (1).

The relative abundance of the various sequence classes can also be estimated from the number of randomly derived clones that fall into each class of M13 minicircle.

As shown in Table II, 55 of the 59 *Eco*RI and *Bam*HI clones obtained were of the KSR1 sequence class, and four were of the F class, 19 of the Lt26 class and 32 were of the Lt154 class, 19 of the Lt26 class and one of the P class. The percentage of *Eco*RI minicircles in class F is consistent with the relative amount of the minor unit-length *Eco*RI band that is retarded in acrylamide, considering the relatively small number of random clones examined so far.

DISCUSSION

We have sequenced three cloned minicircle molecules from the kDNA of *L. tarentolae*. These represent different sequence classes. Each minicircle has a conserved region of 160–270 bp and a variable region. The conserved regions show a sequence ho-

TABLE II

L. tarentolae kDNA minicircle sequence classes from T ladder patterns

Cloning site	Vector	Orientation [a]	T ladder class	Sequence	Number of clones
*Eco*RI	M13mp8	+	A	KSR1	13
	M13mp7	+	A	KSR1	6
	M13mp7	+	F	–	4
	M13mp8	–	C	KSR1	15
	M13mp7	–	C	KSR1	4
*Bam*HI	M13mp8	+	D	KSR1	10
	M13mp8	–	E	KSR1	7
					59 [b]
*Hin*dIII	M13mp8	+	G	Lt154	16
	M13mp8	+	H	Lt26	10
	M13mp8	+	P	–	1
	M13mp8	–	B	Lt154	16
	M13mp8	–	L	Lt26	9
					52 [c]

[a] See diagram in Fig. 10 for explanation of orientation.
[b] The total number of *Eco*RI and *Bam*HI minicircle clones examined.
[c] The total number of *Hin*dIII minicircle clones examined.

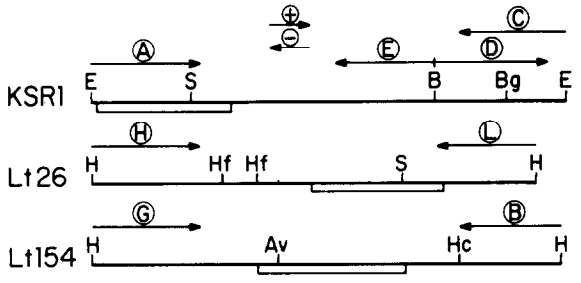

Fig. 10. Diagrams of known sequence classes corresponding to T ladder patterns from data presented in Table II. The orientations of the conserved regions of each sequence are specified arbitrarily by symbols ⊕ and ⊖.

mology of approx. 80%. The conserved region in the *Leishmania* minicircles is larger than that in the *T. brucei* (Chen and Donelson, 1980) and the *T. equiperdum* (Barrois et al., 1982) minicircles, and the *Leishmania* minicircles lack the large number of scattered internal repeats of 10 or more bp present in the *T. brucei* minicircles. The *L. tarentolae* sequences also have frequent termination codons in all reading frames, but as is the case for the *T. brucei* (Chen and Donelson, 1980) sequences, there are short ORFs that are within or near the conserved regions. There is no evidence that these ORFs are functional in terms of transcription and translation.

The relative abundance of these minicircle sequence classes in the network DNA was examined by first determining the frequency of minicircles containing single *Eco*RI, *Bam*HI or *Hin*dIII sites by gel analysis and then by random cloning of unit length minicircles into M13 at these sites and comparison of the T ladder patterns of these random inserts. Most of the T ladder patterns corresponded to the known minicircle sequences but, in addition, two new patterns, F and P, were observed. The sequence organization of these minor minicircle classes has not been studied. However, they both contain a region homologous to a portion of the sequenced minicircles, since both class F and class P M13 phage clones can form gel-retarded hybrids with mKSR1 phage DNA and show cross hybridization with the unit length *Hin*dIII minicircle bands from total kDNA digests (results not shown).

We conclude that approx. 50% of the minicircles in the network fall into at least five different sequence classes. The KSR1 minicircle sequence class is apparently identical to the Class II minicircle sequence class of Challberg and Englund (1980), since the revised sequence (Englund, P., personal communication) of the *Mbo*I fragment of Class II minicircles from *L. tarentolae* is identical to that portion of the KSR1 sequence. It should be noted that the T ladder patterns are useful only for distinguishing overall patterns of sequences and would not distinguish single-base changes. However, two independently cloned minicircles, those in pLt19 and pKSR1, had identical sequences. Furthermore redigestion with *Msp*I and *Hae*III of gel-purified *Bam*HI and *Eco*RI minicircle bands released from total kinetoplast DNA each gave the banding pattern expected for a homogeneous species. Thus, we conclude that at least the KSR1 sequence class, which represents 26% of the total minicircle DNA, is homogeneous. Our data do not allow any conclusions to be made regarding the homogeneity of the Lt26 and Lt154 sequence classes, but T ladders from several independent clones show no detectable heterogeneity.

The organization of minicircle sequences into a conserved and variable region seems to be general among the kinetoplastid protozoa, although in the case of *T. cruzi* (Van Heuverswyn et al., 1982) the conserved region is present in a fourfold repeat. In nature, minicircle sequences in *Leishmania* sp (Simpson et al., 1980; Wirth and Pratt, 1982; Arnot and Barker, 1981), *T. cruzi* (Morel et al., 1980; Frasch et al., 1981) and *T. brucei* (Steinert et al., 1976; Borst et al., 1980c) are known to change rapidly, and a few sequence changes have been observed in cultured *L. tarentolae* (Simpson et al., 1980) and *Crithidia* (Hoeijmakers and Borst, 1982) in a several-year period. However, nothing is known about the relative rates of change of the conserved and variable regions in any one species or strain. There is some evidence for recombination between minicircles of *Crithidia* from density-shift experiments (Manning and Wolstenholme, 1976) and electron microscopy of heteroduplexes (Hoeijmakers et al., 1982). The question arises as to the mechanism for the maintenance of separate homogeneous minicircle sequence

Anomalous electrophoretic migration of certain minicircle fragments has been observed with *L. tarentolae* kDNA (Simpson et al., 1980; Marini et al., 1982) and *T. brucei* kDNA (Chen and Donelson, 1980) and may be a general phenomenon in all kinetoplastids. We have shown that this property is associated with the conserved region of the minicircle and

can be destroyed, at least in one case, by a single cleavage at an appropriate site. Marini et al. (1982) have proposed that this property is due to a bend in the DNA molecule that is a function of the sequence and they have speculated that this bend may play a role in the packaging of the minicircle DNA in the kinetoplast nucleoid body or as a recognition site for DNA-binding proteins.

The function of the minicircle DNA remains unknown. The existence of small ORFs within the constant regions in two species is intriguing and deserves further study. In this regard, the absence of an ORF of equivalent or greater size in the minicircle DNA of *T. equiperdum* (Barrois et al., 1982), a species that has a permanently nonfunctional mitochondrion associated with a deleted maxicircle DNA (in one strain) and a homogeneous minicircle, may imply a transcriptional role for the minicircle, perhaps associated with the transition to that stage in the life cycle in which the mitochondrion is functional.

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