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The majority of minicircle DNA in *Crithidia fasciculata* strain CF-C1 is of a single class with nearly homogeneous DNA sequence

Larry Birkenmeyer, Hiroyuki Sugisaki and Dan S.Ray*

Department of Biology, and Molecular Biology Institute, University of California, Los Angeles, CA 90024, USA

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ABSTRACT

DNA minicircles found within the kinetoplast of the trypanosomatid Crithidia fasciculata, like those of most other kinetoplastid species, are heterogeneous in sequence. The pattern of minicircle DNA fragments generated by cleavage of kinetoplast DNA with various restriction enzymes has been used to demonstrate this heterogeneity. Here we describe a strain of Crithidia fasciculata in which more than 90% of the DNA minicircles exhibit a common pattern of restriction enzyme cleavage sites. A map of cleavage sites within this major minicircle DNA class is presented for seven restriction enzymes with hexanucleotide recognition sequences. Sequence homogeneity at an even finer level is reflected in minicircle DNA digestion patterns generated by restriction enzymes with tetranucleotide recognition sites. Partial DNA sequence analysis of multiple clones from the major minicircle class shows nearly complete homogeneity at the nucleotide level. The existence of a near homogeneous complement of DNA minicircles in Crithidia should facilitate the study of their replication in this organism.

INTRODUCTION

Trypanosomes contain a single mitochondrion termed the kinetoplast. Two distinct populations of double-stranded circular DNA, the maxicircles and minicircles, are localized within the kinetoplast as a single highly catenated DNA network known as kinetoplast DNA (kDNA) (for reviews see refs. 1-4). In contrast to the maxicircle DNA (the trypanosome equivalent of conventional mitochondrial DNA), minicircle DNA of most trypanosomes is heterogeneous in sequence (2,3). Instances in which a species contains homogeneous minicircles correlate with the absence of or defects in maxicircle DNA (5,6). This has suggested the involvement of maxicircle DNA in the maintenace of minicircle DNA heterogeneity (5,7). In the trypanosomatid Crithidia fasciculata each kDNA network contains about 5000 DNA minicircles (2.5 kb) and about 40 DNA maxicircles (37 kb) (2,8). DNA renaturation kinetics and restriction enzyme cleavage analysis has shown the minicircle population in this species to be heterogeneous in sequence (2,9-12).

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A strain of Crithidia fasciculata containing a complement of homogeneous minicircles would simplify the analysis of their replication and make possible sequence analysis on a major portion of the minicircle DNA of the cell. Such a strain is described here. Greater than 90% of the minicircles from the strain C. fasciculata Cf-C1 are homogeneous; a result obtained through cleavage analysis of kDNA with various restriction enzymes and DNA sequence analysis. A restriction enzyme cleavage map of this homogeneous minicircle DNA population is presented here along with partial DNA sequence data from multiple independent clones of half-length XhoI-StuI fragments of the major minicircle class.

MATERIALS AND METHODS

Cell Growth

Crithidia fasciculata Cf-C1 cells (13) were cultured in Brain Heart Infusion medium (Difco) supplemented with 10 μg/ml Hemin (Sigma) and 100 μg/ml streptomycin sulfate (Sigma) at 28°C with shaking.

Kinetoplast DNA and Restriction Enzymes

Kinetoplast DNA was prepared from stationary phase cultures and purified on cesium chloride-ethidium bromide density gradients as described (13). Restriction endonucleases were purchased from Boehringer-Mannheim (AluI, DraI, RsaI, StuI, XhoI), New England Biolabs (BssHII, MluI, MspI, XbaI), Bethesda Research Laboratories (BalI, HhaI), and International Biotechnologies, Inc. (SacII).

Gel Electrophoresis

Digested kDNA samples were electrophoresed in 0.8% and 2.0% agarose horizontal slab gels in a Tris-borate-EDTA buffer plus ethidium bromide (EtBr) as previously described (14). DNA gel electrophoresis markers were obtained from Bethesda Research Laboratories.

Cloning Half-Length Minicricle Fragments

The double-stranded replicated form (RF) of the M13 vectors mp10 and mp11 were prepared as described (15). Each RF was digested with SmaI and SalI restriction enzymes purchased from Bethesda Research Laboratories. The linear vector DNAs were purified by gel filtration on a Bio-Gel A-5m column (Bio-Rad Laboratories). Purified kinetoplast DNA was digested with XhoI and StuI and ligated to each linear vector DNA. Transfection and purification methods for recombinant phages were as published (15). RF DNA was prepared from independent isolates and the identity and orientation of the insert determined by restriction enzyme analysis. The two half-length

minicircle fragments were cloned into mp11 with equal frequency. However, in the case of mp10 the half-length fragment containing the single SacII site was obtained in only 10% of the recombinants. Additional isolates of this class were obtained for comparison by cloning first into mp11 and then transferring the inserted fragment into mp10. These isolates are indicated by the letter C following the isolate number.

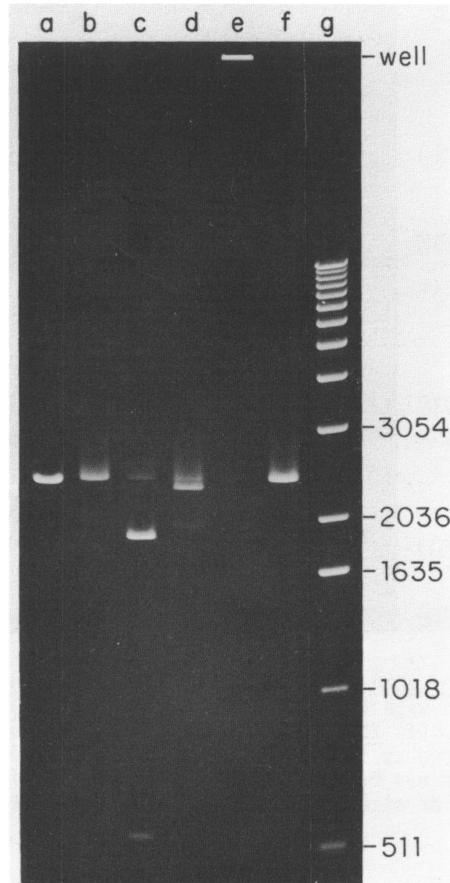


Figure 1. Cleavage of kDNA by restriction endonucleases recognizing hexanucleotide sites. kDNA (0.25 µg) from Crithidia fasciculata LA-1 was digested with (a) XhoI, (b) SacII, (c) DraI, (d) BalI, (e) BssHII or (f) MluI for 2 hrs at 37°C, except for the BssHII reaction which was incubated at 50°C. Each digest was electrophoresed in a 0.8% agarose gel (see Materials and Methods). In lane (d) a small BalI fragment has been run off the gel. Linear DNA size standards (indicated in base pairs) are shown at the right (lane g).

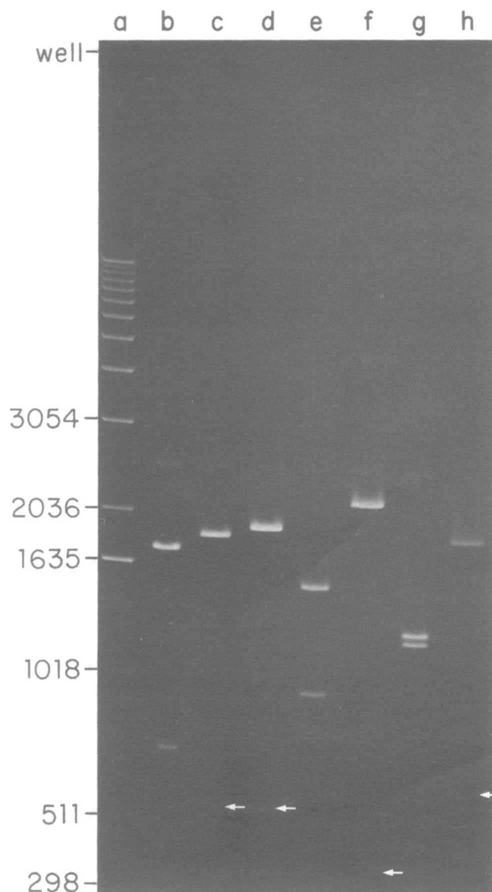


Figure 2. Digestion of kDNA by pairs of restriction endonucleases. kDNA (0.25 μg) was digested with (b) XhoI + SacII, (c) XhoI + DraI, (d) XbaI + DraI, (e) XhoI + MluI, (f) XbaI + MluI, (g) XhoI + StuI, and (h) XbaI + StuI and electrophoresed as in Figure 1. In each of the digests involving DraI, a small fragment has been run off the gel. Arrows indicate the positions of the smaller size fragments. Size standards are shown on the left (lane a).

Sequencing

DNA sequencing reactions were performed by the dideoxy chain termination method (16). Synthesis was primed with the universal 15 nucleotide primer (Takara Shuzo Co.) complementary to a region of the phage vector sequence immediately upstream of the cluster of cloning sites.

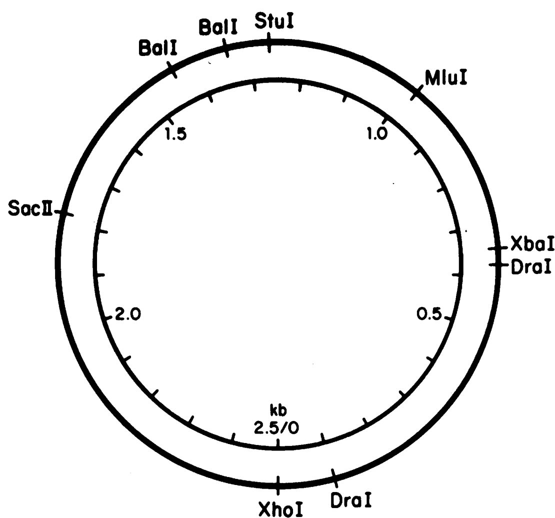


Figure 3. Restriction endonuclease cleavage map of the major minicircle DNA species. Sites of cleavage for the indicated restriction enzymes are shown on the outer circle. The inner circle scale is presented in kilobase pairs.

RESULTS

Cleavage of kDNA by Restriction Endonucleases with Hexanucleotide Recognition Sites

Kitchin *et al*. (17,18) reported recently that the restriction endonucleases *Sst*II and *Xho*I each make a single cleavage in virtually all DNA minicircles found in the kDNA of *Crithidia fasciculata*. We have tested other enzymes for similar cleavage of kDNA. Besides *Sac*II (an isoschizomer of *Sst*II) and *Xho*I, five additional enzymes with hexanucleotide recognition sites were found which cleaved nearly all minicircles either once (*Mlu*I, *Stu*I, *Xba*I) or twice (*Bal*I, *Dra*I). In Figure 1 the extent of kDNA cleavage by several of these enzymes is shown in comparison to *Bss*HII which shows no detectable cutting. Judging by the lack of EtBr staining material trapped in the wells and the barely detectable levels of uncut free minicircles, we estimate that more than 90% of the minicircles are cleaved by these enzymes. Minor amounts of minicircle DNA were released from the kDNA network by *Eco*RI, *Bam*HI, and *Pvu*II digestion (data not shown).

Spacing Between Cleavage Sites

Two unique size fragments were generated upon *Dra*I digestion of kDNA (Fig. 1, lane c), the sum of which equals the expected 2.5 kb unit length

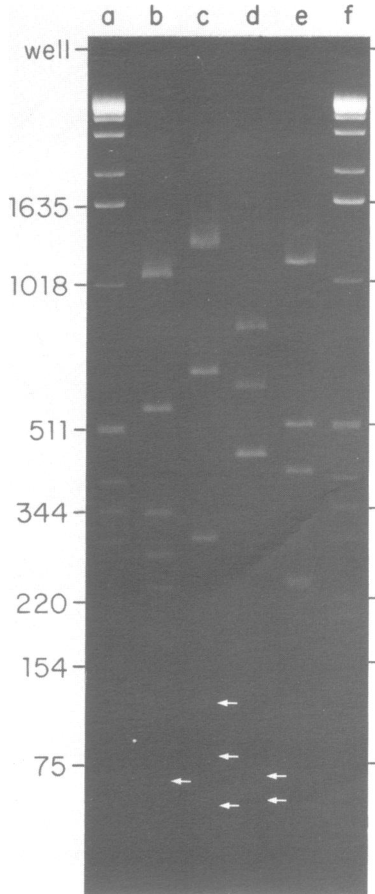
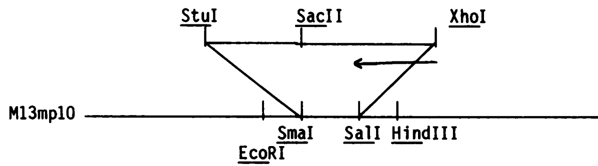
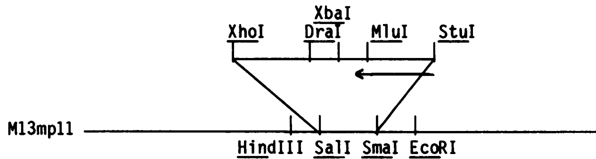


Figure 4. Cleavage of kDNA by restriction endonucleases recognizing tetranucleotide sites. kDNA (0.65 μg) was digested with (b) AluI, (c) MspI, (d) HhaI, or (e) RsaI for 2 hrs at 37°C. The digests were electrophoresed on a 2.0% agarose gel (see Materials and Methods). Arrows indicate the position of smaller size fragments. Size standards are shown in lanes (a) and (f).

minicircle; thus the spacing of DraI cleavage sites is conserved among the majority of minicircles. Double digests were performed with various combinations of enzymes to determine whether or not the spacing between heterologous cleavage sites had also been preserved. Figure 2 presents the banding patterns generated by several such double digests. The sizes of the major fragments seen in each lane add up to 2.5 kb; similar results were obtained for digests involving XbaI + SacII, XhoI + BalI, and XbaI



CLONES: M13CFK41, 55, 62, 64, 81, 102C, 114C, 128C, 141C, 133C



CLONES: M13CFK111, 112, 118, 120, 123, 129, 131, 136, 146

Figure 5. Regions sequenced in independent clones of half-length fragments of minicircles. The upper and lower maps show the orientation of the StuI-XhoI fragments used for sequence analysis. The arrows indicate the regions sequenced in each set of isolates. Maps are not to scale.

+ BalI (data not shown). A small portion of the minicircles in each lane remain at the position of linear minicircle DNA. This may be due to minor populations of minicircle DNA cleaved by one enzyme but not the other, or a result of incomplete digestion. Based on the results of the double digestion experiments, a restriction enzyme cleavage map for the major minicircle DNA population was derived (Fig. 3).

In light of previous evidence demonstrating minicircle DNA heterogeneity in *Crithidia fasciculata*, our results were unexpected. Verification that the cells used in our studies were of the same species came from the characteristic minicircle size and the diagnostic pattern of maxicircle DNA cleavage fragments (19) produced by several restriction enzymes (data not shown).

Figure 4 shows the minicircle DNA fragment patterns obtained upon digestion of kDNA with restriction enzymes having tetranucleotide recognition sites. They differ from the reported cleavage patterns (10) in that the overall number of different size DNA fragments is dramatically reduced and the fragments appear in approximately stoichiometric amounts. Extended electrophoresis (unpublished results) and staining intensity indicate that the 450 base pair (bp) HhaI band (lane d) and the 250 bp RsaI band (lane e) are actually doublets. If the few faint non-stoichiometric

bands are ignored, the sum of the fragment sizes within each lane is very close to the full length 2.5 kb minicircle (±50 base pairs). Thus, at the somewhat finer level of resolution afforded by these restriction enzymes due to their more frequent cleavage, the majority of the kDNA minicircles still appear homogeneous.

Comparison of Minicircle DNA Sequences

To examine the degree of homogeneity of the major minicircle class at

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              10         20         30         40         50         60         70
 81   TCGAGTGCGA TGTTGTGTTG ATAGCTTCTT GTAGTTTTTC GTTGTTTGTT AATGTTGGTG TTGGTGTTGG
128C  .......... .......... .......... .......... .......... .......... ..........
141C  .......... .......... .......... .......... .......... .......... ..........
133C  .......... .......... .......... .......... .......... .......... ..........
 41   .......... .......... .......... .......... .......... .......... ..........
 55   .......... .......... .......... .......... .......... .......... ..........
 62   .......... .......... .......... .......... .......... .......... ..........
 64   .......... .......... .......... .......... .......... .......... ..........
102C  .......... .......... .......... .......... .......... .......... ..........
114C  .......... .......... .......... .......... .......... .......... ..........

              80         90        100        110        120        130        140
 81   TGTTCTCGGT TGCCACCTGT GGTTTCTTTA AGTGTTTGTT GCTGTTTATT TTGTTGTTTG TTGGTTATTG
128C  .......... .......... .......... .......... .......... .......... ..........
141C  .......... .......... .......... .......... .......... .......... ..........
133C  .......... .......... .......... .......... .......... .......... ..........
 41   .......... .C........ N......... .......... .......... .......... ..........
 55   .......... .......... N......... .......... .......... .......... ..........
 62   .......... .......... N......... .......... .......... .......... ..........
 64   .......... .......... N......... .......... .......... .......... ..........
102C  .......... .A........ .......... .......... .......... .......... ..........
114C  .......... .......... .......... .......... .......... .......... ..........

             150        160        170        180        190        200        210
 81   GTTTATTGTT TGCATTAGCC TTCTGTGGGT TTGAAACTGT TGTATTCTTG TTTACTTGGG TGGTTTATCT
128   .......... .......... .......... .......... .......... .......... ..........
141C  .......... .......... .......... .......... .......... .......... ..........
133C  .......... .......... .......... .......... .......... .......... ..........
 41   .......... .......... .......... .......... .......... .......... ..........
 55   .......... .......... .......... .......... .......... .......... ..........
 62   .......... .......... .......... .......... .......... .......... ..........
 64   .......... .......... .......... .......... .......... .......... ..........
102C  .......... .......... .......... .......... .......... .......... ..........
114C  .......... .......... .......... .......... .......... .......... ..........

             220        230        240        250        260        270        280
 81   TGATTTGGCT TTATTGTTGG GTACTTGTTG TTGTTTGTTG TGTTTTATGC TGTTTCTTTG TTGCTGGTGC
128C  .......... .......... .......... .......... .......... .......... ..........
141C  .......... .......... .......... .......... .......... .......... ..........
133C  .......... .......... .......... .......... .......... .......... ..........
 41   .......... .......... .......... .......... .......... .......... ..........
 55   .......... .......... .......... .......... .......... .......... ..........
 62   .......... .......... .......... .......... .......... .......... ..........
 64   .......... .......... .......... .......... .......... .......... ..........
102C  .......... .......... .......... .......... .......... .......... ..........
114C  .......... .......... .......... .......... .......... .......... ..........

             290        300        310        320        330        340       349
 81   TTGCTGAACT GTTTGTGGTT CGTTGGGGCG TGTGGGTTTG AGGGTGTTTT TTGGGGTGGT TTGGGGTGC
128C  .......... .......... .......... .......... .......... .......... .........
141C  .......... .......... .......... .......... .......... .......... .........
133C  .......... .......... .......... .......... .......... .......... .........
 41   .......... .......... .......... .......... .......... .......... .........
 55   .......... .......... .......... .......... .......... .......... .........
 62   .......... .......... .......... .......... .......... .......... .........
 64   .......... .......... .......... .......... .......... .......... .........
102C  .......... .......... .......... .......... .......... .......... .........
114C  .......... .......... .......... .......... .......... .......... .........
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             10         20         30         40         50         60         70
111  CCTCCTGGCA GGGGGTTTGG CGGGGTTCTA GCCCGATTTC GGGGCGTTCT GCGGGGGTTT TTTCTGGTCT
112  .......... .......... .......... .......... .......... .......... ..........
118  .......... .......... .......... .......... .......... .......... ..........
120  .......... .......... .......... .......... .......... .......(   T7).......
123  .......... .......... .......... .......... .......... .......... ..........
129  .......... .......... .......... .......... .......... .......(   T5).......
131  .......... .......... .......... .......... .......... .......... ..........
136  .......... .......... .......... .......... .......... .......... ..........
146  .......... .......... .......... .......... .......... .......... ..........

             80         90        100        110        120        130        140
111  GGGCGCGGGT TTGGGCTGGT TTGGGCTGGG TTTGGACTGT TTGTGCTAGT TGGGCGCTAC GGACTGCTTT
112  .......... .......... .......... .......... .......... .......... ..........
118  .......... .......... .......... .......... .......... .......... ..........
120  .......... .......... .......... .......... .......... .......... ..........
123  .......... .......... .......... .......... .......... .......... ..........
129  .......... .......... .......... .......... .......... .......... ..........
131  .......... .......... .......... .......... .......... .......... ..........
136  .......... .......... .......... .......... .......... .......... ..........
146  .......... .......... .......... .......... .......... .......... ..........

            150        160        170        180        190        200        210
111  GCGATGGTGC GCGGGGGGTG GTTTCACCAC TATTCTGATT GTTGTTTTCG CTCCTTGGTG GGGTTTATAT
112  .......... .......... .......... .......... .......... .......... ..........
118  .......... .......... .......... .......... .......... .......... ..........
120  .......... ..( G7 ).. .......... .......... .......... .......... ..........
123  .......... ..( G7 ).. .......... .......... .......... .......... ..........
129  .......... .......... .......... .......... .......... .......... ..........
131  .......... ..( G7 ).. .......... .......... .......... .......... ..........
136  .......... .......... .......... .......... .......... .......... ..........
146  .......... .......... .......... .......... .......... .......... ..........

            220        230        240        250        260        270        280
111  GCGCTCCGTT CGGTCGTATT CTGGAATTTT GGGGTTTGCA AAAGTGACTT CCGGCATTTC TCGCGGGGTT
112  .......... .......... .......... .......... .......... .......... ..........
118  .......... .......... .......... .......... .......... .......... ..........
120  .......... .......... .......... .......... .......... .......... ..........
123  .......... .......... .......... .......... .......... .......... ..........
129  .......... .......... .......... .......... .......... .......... ..........
131  .......... .......... .......... .......... .......... .......... ..........
136  .......... .......... .......... .......... .......... .......... ..........
146  .......... .......... .......... .......... .......... .......... ..........

            290        300        310        320        330        340        350
111  AATATATAGA CTAGACGCGT CGTTGTTAAT TTTGCCATGA GTGTGTTTGT GGTTGTTCTG GTCCCGGAGT
118  .......... .......... .......... .........G .........G T......... ..
120  .......... .......... .......... .........G .........G T......... ..
123  .......... .........C .......... .........G .........G T......... ..
129  .......... .......... .......... .........G .......... .
131  .......... .......... .......... .........G .......... .......... ..
136  .......... .......... .......... .........G .........G T......... ..
146  .......... .......... .......... .........G .........G T......... ..

            360        370        380        390        399
111  GATTTGGCCC CTCCCGCAAA AATAGAAACG GTCTGGGTAG GGGCGTTCT
112
118
120
123
129
131
136
146
```

Figure 6. DNA sequences of the minicircle regions indicated in Fig. 5. Identical nucleotides are indicated by dots. Uncertain bases are indicated by the letter N.

the nucleotide level we have cloned XhoI-StuI half-length molecules (Fig. 5) into the M13 vectors mp10 and mp11. Whole molecules of this class were found to be very unstable in both M13 and pUC vectors. Partial DNA sequences from multiple independent isolates of each half molecule are presented in Fig. 6. The two regions sequenced are shown in Fig. 5 and together account for approximately 30% of the entire 2.5 kb minicircle. Sequence differences among independent clones are limited to a very few single base additions, deletions, or substitutions. Most often the addition or deletion of a base is simply the result of heterogeneity in the number of bases within a stretch of identical bases. However, it is the extent of nucleotide sequence conservation in the two minicircle regions analyzed, not the slight sequence microheterogeneity, that is the most striking.

DISCUSSION

Evidence reflecting minicircle DNA heterogeneity in *Crithidia fasciculata* has been reported (9-12). The strain described here, Cf-C1, contains one major class of DNA minicircles as judged by restriction enzyme cleavage analysis. There are minor minicircle classes which are cleaved by restriction enzymes that do not recognize the major class. However, these minor classes are estimated to contain less than 10% of the total number of minicircles.

DNA sequence analysis of two separate minicircle regions demonstrate that the major minicircle class is close to homogeneous at the nucleotide level, at least within these regions. The sequence data along with the restriction enzyme cleavage analysis, strongly suggests that the majority of minicircles in this strain are nearly identical in DNA sequence. Other regions of the major minicircle class are currently being sequenced.

The relationship of the Cf-C1 strain to strains of *Crithidia fasciculata* in use in other laboratories is not yet known. *C. fasciculata* Cf-C1 has been maintained in our laboratory for the past four years after having being obtained from the laboratory of Dr. Larry Simpson at UCLA. This strain was colony purified more than fourteen years ago and has been propagated from single colony isolates several times since then. It will be of interest to examine other strains of *Crithidia fasciculata* for the presence and relative amount of this nearly homogeneous minicircle population. Such a homogeneous population could have been obscured in other studies by the large amounts of kDNA commonly used for restriction enzyme cleavage analysis in order to accentuate the presence of the minor

minicircle classes.

A correlation has been drawn between the presence of a functional maxicircle and the maintenance of minicircle heterogeneity (5,6). Restriction enzyme cleavage analysis of the maxicircle DNA from the strain used in this study revealed no gross structural anomalies (unpublished results) and confirmed the species identity as Crithidia fasciculata. However, such an analysis does not rule out other possible maxicircle defects.

Crithidia fasciculata is often used as a model system for studying kDNA replication. A strain containing a near homogeneous complement of DNA minicircles should facilitate the identification of minicircle replication intermediates and make possible mapping of the minicircle DNA origin of replication and other features of possible biological significance.

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*To whom correspondence should be addressed

REFERENCES

1. Simpson, L. (1972) Int. Rev. Cytol. 32, 139-207.
2. Borst, P., and Hoeijmakers, J.H.J. (1979) Plasmid 2, 20-40.
3. Englund, P.T. (1981) in Biochemistry and Physiology of Protozoa, Levandowsky, M., and Hutner, S.H. Eds., 2nd Edition, Vol. 4, pp. 333-383, Academic Press, New York.
4. Englund, P.T., Hajduk, S.L., and Marini, J.C. (1982) Ann. Rev. Biochem. 51, 695-726.
5. Borst, P., and Hoeijmakers, J.H.J. (1979) in Extrachromosomal DNA. ICN-UCLA Symposia on Molecular and Cellular Biology, Cummings, D.J., Borst, P., David, I.B, Weissman, S.M., and Fox, C.F. Eds., Vol. 15, pp. 515-531, Academic Press, New York.
6. Frasch, A.C.C., Hajduk, S.L., Hoeijmakers, J.H.J., Borst, P., Brunel, F., and Davison, J. (1980) Biochim. Biophys. Acta 607, 397-410.
7. Hoeijmakers, J.H.J., Weijers, P.J., Brakenhoff, G.J., and Borst, P. (1982) Plasmid 7, 221-229.
8. Englund, P.T. (1978) Cell 14, 157-168.
9. Steinert, M., and van Assel, S. (1980) Plasmid 3, 7-17.
10. Hoeijmakers, J.H.J., and Borst, P. (1982) Plasmid 7, 210-220.
11. Brack, C., Bickle, T.A., Yaun, R., Barker, D.C., Foulkes, M., Newton, B.A., and Jenni L. (1976) in Biochemistry of Parasites and Host-Parasite Relationships, van den Bossche, H. Ed., pp. 211-218, North Holland Publishing Company, Amsterdam.
12. Borst, P., Fase-Fowler, F., Steinert, M., and van Assel, S. (1977) Exp. Cell Res. 110, 167-173.
13. Simpson, A.M., and Simpson, L. (1974) J. Protozool. 21, 774-781.
14. Sugden, B., De Troy, B., Roberts, R.J., and Sambrook, J. (1975) Anal. Biochem. 68, 36-46.

15. Messing, J. (1983) Methods in Enzymology 101, 20-89.
16. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
17. Kitchin, P.A., Klein, V.A., Fein, B.I., and Englund, P.T. (1984) J. Biol. Chem. 259, 15532-15539.
18. Kitchin, P.A., Klein, V.A. and Englund, P.T. (1985) J. Biol. Chem. 260, 3844-3851.
19. Hoeijmakers, J.H.J., Schoutsen, B., and Borst, P. (1982) Plasmid 7, 199-209.