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kDNA minicircles of the major sequence class of *C. fasciculata* contain a single region of bent helix widely separated from the two origins of replication

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ABSTRACT

The major sequence class of Crithidia fasciculata minicircles is shown to have a single region of bent helical DNA widely separated from the two replication origins located 180° apart on the minicircle map. The position of the bend in the DNA has been mapped both by gel electrophoretic methods and by direct electron microscopic observation of the DNA. This sequence directed bending is apparently the result of homopolymeric dA•dT tracts 4-6 base pairs long repeated in phase with the helix screw. The region of the bend contains nineteen such homopolymeric tracts in a region of about 200 base pairs with sixteen of the tracts oriented in the same direction.

INTRODUCTION

The kinetoplast DNA (kDNA) of trypanosome species is an enormous network of catenated DNA molecules (1) containing thousands of small circular DNA molecules (minicircles) and about 20-50 larger circular DNA molecules (maxicircles). The maxicircles contain the usual mitochondrial genes and correspond to the mitochondrial genomes of other organisms (2). On the other hand, the minicircles have no analog in other organisms and appear to represent a high copy number mitochondrial plasmid with no known function.

The kinetoplast minicircles are unusual in still other ways. In most trypanosome species the minicircles show a high degree of sequence heterogeneity (3-5). Outside of a conserved region of about 100-200 base pairs the nucleotide sequence shows a high degree of variability between individual molecules. In contrast, the minicircles of the African trypanosome T. equiperdum appear to be homogeneous in sequence (6), and those of the major sequence class of the trypanosomatid Crithidia fasciculata are nearly homogeneous (7). Comparison of the nucleotide sequences of all minicircles sequenced to date shows only a twelve nucleotide sequence common to all minicircles. In all cases this

"universal minicircle sequence" is contained within the larger (100-200 bp) conserved sequence region. In newly replicated minicircles of T. equiperdum (8) and C. fasciculata (Birkenmeyer, Sugisaki and Ray, unpublished), the universal minicircle sequence overlaps the 5' terminus of a specific gap in one strand of the minicircle. While minicircles from most species contain only a single copy of the conserved region, T. lewisi (9) and C. fasciculata (Sugisaki and Ray, unpublished) minicircles both contain two copies of their conserved regions located 180° apart on the minicircle.

Minicircles of most trypanosomes also contain a region of bent helix (10-13). This feature of minicircle DNA appears to represent a static bend in the DNA induced as a consequence of repeated stretches of homopolymeric dA·dT sequences 4-6 base pairs long repeated in phase with the helix screw (14,15). This sequence-directed bending of the DNA helix results in an anamolously slow electrophoretic mobility of DNA fragments in acrylamide gels (16).

Fragments having the most anamolous migration of any observed so far have been derived from C. fasciculata minicircles (13). Since there are two copies of the conserved sequence region in C. fasciculata, it was of interest to determine the number of regions of bent helix and their location(s) relative to the conserved sequences. In this work we show that Crithidia minicircles of the major class contain a single region of bent helix which is widely separated from the two origins of replication.

MATERIALS AND METHODS

Restriction Enzymes and Recombinant DNA Methods

Restriction enzymes, polynucleotide kinase, Klenow fragment of polymerase I, calf intestinal phosphatase and T4 DNA ligase obtained from Bethesda Research Labs, Boehringer-Mannheim, New England BioLabs or Pharmacia P-L were used according to the manufacturers' instructions. Unless indicated otherwise, all recombinant DNA methods are described by Maniatis (17).

Cell Growth and Kinetoplast DNA Isolation

Growth of Crithidia fasciculata strain Cf-C1 and isolation of kDNA networks was as described (18).

Mapping the Bent Helix in kDNA Minicircles

The bent helical region in kDNA minicircles was mapped by first digesting total kDNA with either XhoI or MluI followed by end labeling with

^{32}P as described (17). Molecules end labeled at the single XhoI site were then digested with either DraI or AvaI to give one large labeled fragment and one small labeled fragment. The maxicircle fragments and minor sequence classes of minicircles are in such low abundance in such digests that their presence does not interfere with the analysis of the major sequence class of minicircles. Digestion with additional restriction enzymes, one at a time, was carried out to give a progressive shortening of the larger minicircle fragment in each case. Molecules end labeled at the single MluI site were digested with either StuI or AvaII followed by digestions with additional restriction enzymes, one at a time, to also give progressive shortening of the larger fragment in each case. Each digest was electrophoresed on a 2% agarose gel and on a 6% polyacrylamide gel in TBE buffer (17) in the absence of ethidium bromide. Both gels were dried under vacuum and exposed to Kodak XAR-5 X-ray film at -70° overnight. Apparent molecular weights were calculated for each end-labeled fragment relative to a series of end-labeled molecular weight markers (BRL 1 kb ladder and a TaqI digest of wild-type M13 RF; only fragments of about 1 kb or less were efficiently labeled in the BRL marker mix). Additional markers included BamHI-digested M13 RF (6.4 kb) and EcoRI-digested M13oriC81 RF (12.2 kb). For each minicircle fragment, a ratio was calculated of the molecular weight estimated from the mobility on the acrylamide gel to that estimated from the mobility on the agarose gel.

Circular Permutation Gel Electrophoresis

More precise mapping of the bent helical region of the Crithidia minicircles was obtained by using the circular permutation gel analysis of Wu and Crothers (12). The XhoI-StuI half-length minicircle fragment from the left half of the minicircle (see Fig. 1) was first cloned into M13mp11 between the SalI and SmaI sites. RF DNA from this isolate was prepared and partially digested with TaqI followed by the isolation of a 365 bp fragment spanning the minicircle sequence from a TaqI site at position 2162 to the StuI site at position 1, plus 11 base pairs of the M13mp11 polylinker. This TaqI partial digest fragment was made blunt ended by treatment with the Klenow fragment of DNA polymerase I and then ligated to PstI linkers (CCTGCAGG; Bethesda Research Laboratories). The resulting fragment was cleaved with PstI and cloned into Pst-cleaved M13mp8 RF. Plaques containing the inserted fragment were identified by hybridization to a nick-translated minicircle DNA probe (19). Positive plaques were picked and used to infect small cultures which were then screened by a minilysate

procedure for isolates containing two inserted copies of the cloned fragment. One isolate (M13bh1) was shown by restriction digestion to contain the minicircle fragment as a tandem dimer. RF DNA from this isolate was used for the circular permutation gel analysis. Cleavage of this DNA with any enzyme that cuts only once within the monomeric minicircle fragment releases a 373 base pair fragment containing the bent helix at varying distances from either end of the fragment depending on the enzyme used. Individual digests were electrophoresed on a 6% polyacrylamide gel and then transferred to nitrocellulose paper in a bidirectional transfer (20). One blot was probed with nick-translated M13 RF DNA and the other was probed with nick-translated minicircle DNA. The released 373 base pair fragment was identified based on its hybridization to minicircle DNA but not to M13 RF.

DNA Sequencing

The complete sequence of the major sequence class of Crithidia minicircles has been determined by sequencing several XhoI-StuI half-length minicircle fragments cloned into M13 vectors. Inserts were sequenced by the dideoxynucleotide chain termination method (21) using either a universal M13 sequencing primer or oligonucleotide primers based on known minicircle sequence information (Sugisaki and Ray, unpublished).

Electronmicroscopy

DNA samples were prepared for microscopy by the aqueous spreading method of Kleinschmidt (22) and examined in a Zeiss EM109 electron microscope which was calibrated using a diffraction grating. Length measurements of DNA molecules were determined using a Hewlett Packard 9864A digitizer and pad.

RESULTS

Mapping the Bent Helix

Initial localization of the region(s) of bent helix in the minicircle population was accomplished by first digesting kDNA networks with an enzyme (XhoI or MluI) that cuts the major sequence class only once, end labeling the DNA and then digesting it with various restriction enzymes to produce specific fragments for analysis by gel electrophoresis. By first cutting the labeled DNA with an enzyme that cleaves a small fragment off of one end, a large fragment labeled at only one end can then be used for mapping relative to the labeled end. Since a static bend in a DNA fragment has a much greater effect on the mobility of the fragment on acrylamide gels than

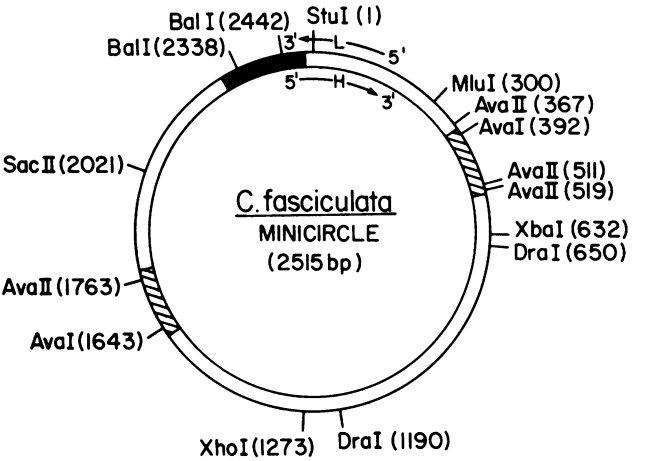

Figure 1. Physical map of the majority sequence class of minicircles. Sites of cleavage by indicated restriction enzymes are shown along with the nucleotide coordinate of the 5' base at each site (7 and unpublished results). The 5' to 3' orientations of the thymine-rich strand (H strand) and the adenine-rich strand (L strand) are shown by arrows. Cross hatched segments indicate the directly repeated sequence common to both major and minor sequence classes. The filled segment indicates the location of the bent helical region identified in this work.

on agarose gels, the presence of a bent helix in a fragment can be detected by determining the mobility of the fragment on an acrylamide gel relative to that on an agarose gel. The position of a bend is located by progressively shortening the fragment and determining the point at which this ratio drops to near unity. The availability of a restriction map for the major class of Crithidia minicircles (Fig. 1) permits the selection of specific restriction enzymes for the sequential shortening of the labeled fragment.

Fig. 2 shows the results of such an experiment using kinetoplast minicircles released from purified networks by cleavage with MluI, end labeling with polynucleotide kinase followed by cleavage with AvaII. The resulting labeled fragments are approximately 67 and 1053 base pairs in length (Fig. 1). Further cleavage of the larger fragment with various enzymes and electrophoresis of each digest on both acrylamide and agarose gels gave the results shown in Fig. 2. Molecular weights were calculated for each fragment relative to a set of marker fragments. The ratio of the calculated molecular weight determined on the acrylamide gel to that determined on the agarose gel for each fragment is shown in Fig. 3(a).

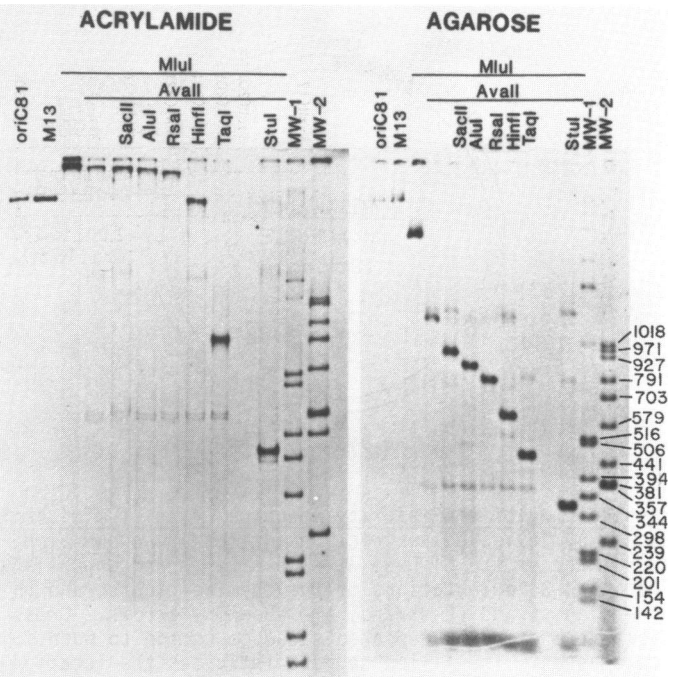

Figure 2. Mapping the bent helix in the majority sequence class of minicircles. Minicircles released from kDNA by MluI digestion and end labeled with ^{32}P were treated with the indicated restriction enzymes and electrophoresed on 2% agarose or 6% acrylamide gels (see Materials and Methods). ^{32}P-labeled markers were Eco RI-cleaved M13oriC81 RF (12.2 kb), BamHI-cleaved M13 RF (6.4 kb), MW-1 (BRL ladder) and MW-2 (TaqI fragments of M13 RF). Sizes of specific marker fragments are indicated adjacent to the agarose gel. Minor bands can be seen in each digest of minicircle DNA due to the presence of fragments derived from minor sequence classes of minicircles and from maxicircle fragments.

Results of a similar experiment in which the second enzyme cleavage of the MluI-cut linear molecules was with StuI are presented on the left-hand side of Fig. 3(a). These results indicate the presence of a single region of bent helix located in the region around the HinfI site at position 2284 and the TaqI site at position 2401. This result was confirmed by experiments using minicircles labeled at the single XhoI site and cleaved with either AvaI or DraI as the second enzyme. These results are shown in Fig. 3(b) and place the bend in the region between the HinfI site at position 2284 and the StuI site at position 1.

The bent helical region was localized more precisely by using the circular permutation gel electrophoresis assay of Wu and Crothers (12). A

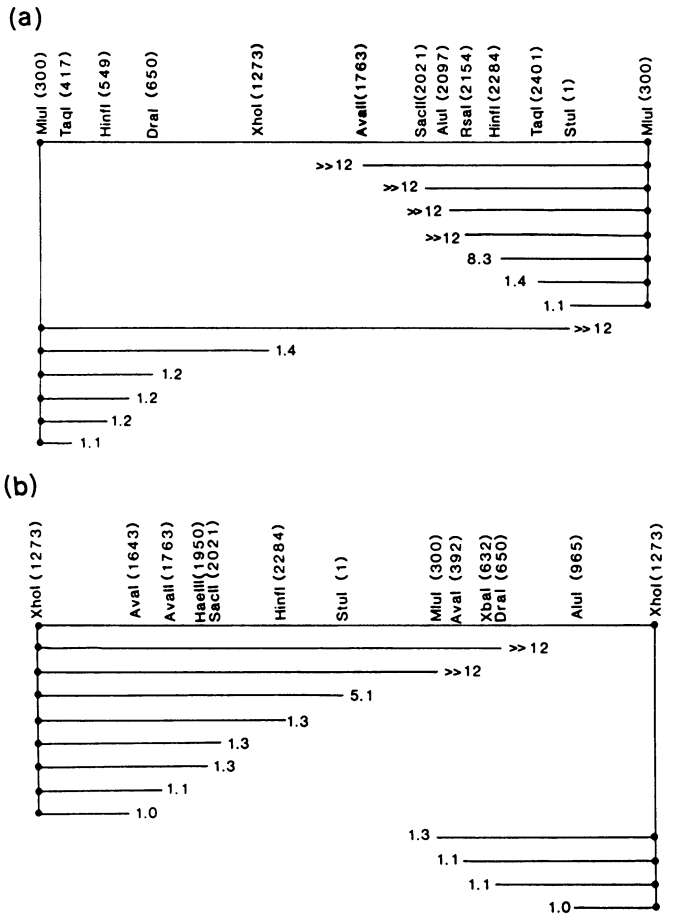

Figure 3. Ratios of fragment sizes estimated on acrylamide gels to those estimated on agarose gels. Relative calculated molecular weights of specific end-labeled minicircle fragments are indicated at the end of each line below a linear representation (not necessarily to scale) of the minicircle map. Filled circles represent the ^{32}P-labeled end of each fragment. Numbers adjacent to each fragment are the calculated ratios of molecular weights determined on the acrylamide gel relative to that determined on the agarose gel. The nucleotide coordinates of selected sites are shown in parentheses. In some lanes of the acrylamide gel the labeled fragment barely enters the gel. In those cases, the molecular weight ratio cannot be determined accurately and is simply indicated as much greater than (>>) 12. (a) Fragments end-labeled at the unique MluI site and (b) fragments end-labeled at the unique XhoI site.

fragment spanning a TaqI site at position 2162 (not shown) to the StuI site at position 1 was cloned into M13mp8 with PstI linkers. Upon screening several isolates, one was found to contain a tandem dimer of the 373

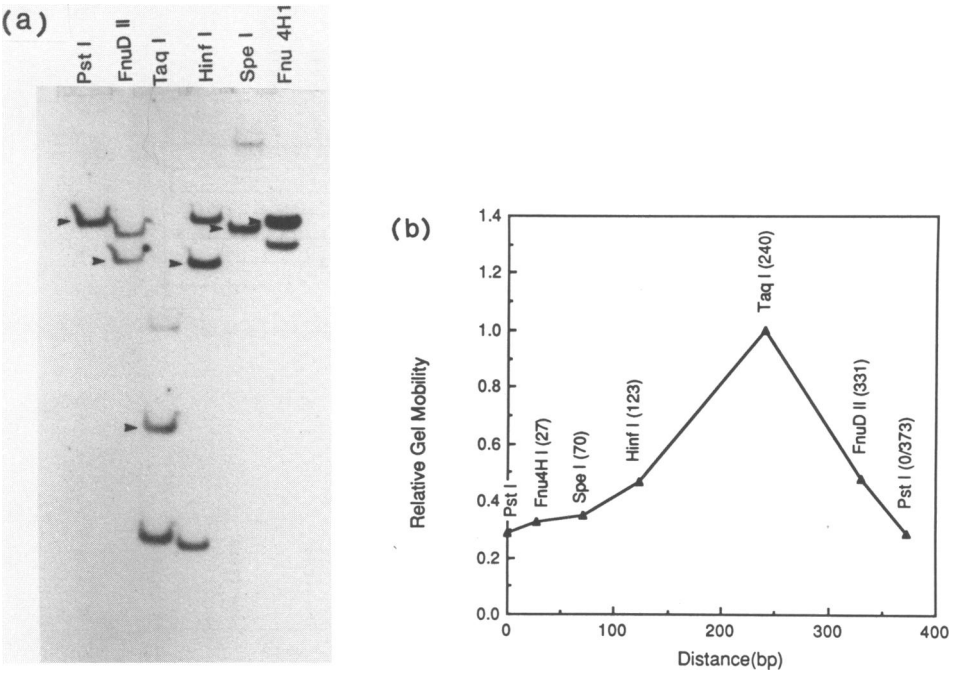

Figure 4. Acrylamide gel electrophoresis of 373 bp minicircle fragments having identical but circularly permuted sequences. Monomeric 373 bp fragments were released from M13bh1 RF containing a 373 bp PstI fragment spanning the bent helical region (see Materials and Methods) which had been cloned into M13mp8 as a tandem dimer. The monomeric minicircle fragment was released by treatment with PstI or with the indicated enzymes that cleave only once within the monomer. (Fnu4HI has two cleavage sites separated by only 3 bp). The monomeric minicircle fragment was identified by performing a bidirectional transfer of the fragments to a pair of nitrocellulose filters and probing one with nick-translated minicircle DNA and the other with nick translated M13 RF. The blot probed with the minicircle probe is shown in (a) and the fragments hybridizing only with the minicircle probe are indicated by arrows. The blot probed with the M13 RF is not shown. The relative gel mobility of each 373 bp minicircle fragment is plotted in (b) as a function of distance from one end of the fragment. The TaqI site at 240 bp from one end corresponds to a TaqI site at position 2401 on the minicircle map located between the BalI sites at positions 2338 and 2442 (see Fig. 1).

base-pair monomeric fragment. Cleavage by various restriction enzymes that cleave within the monomer only a single time releases a 373 base-pair fragment having the bent helix located at different positions depending on which enzyme was used. A cleavage that cuts near the center of the bend is expected to give the fragment with the highest mobility (i.e. the lowest

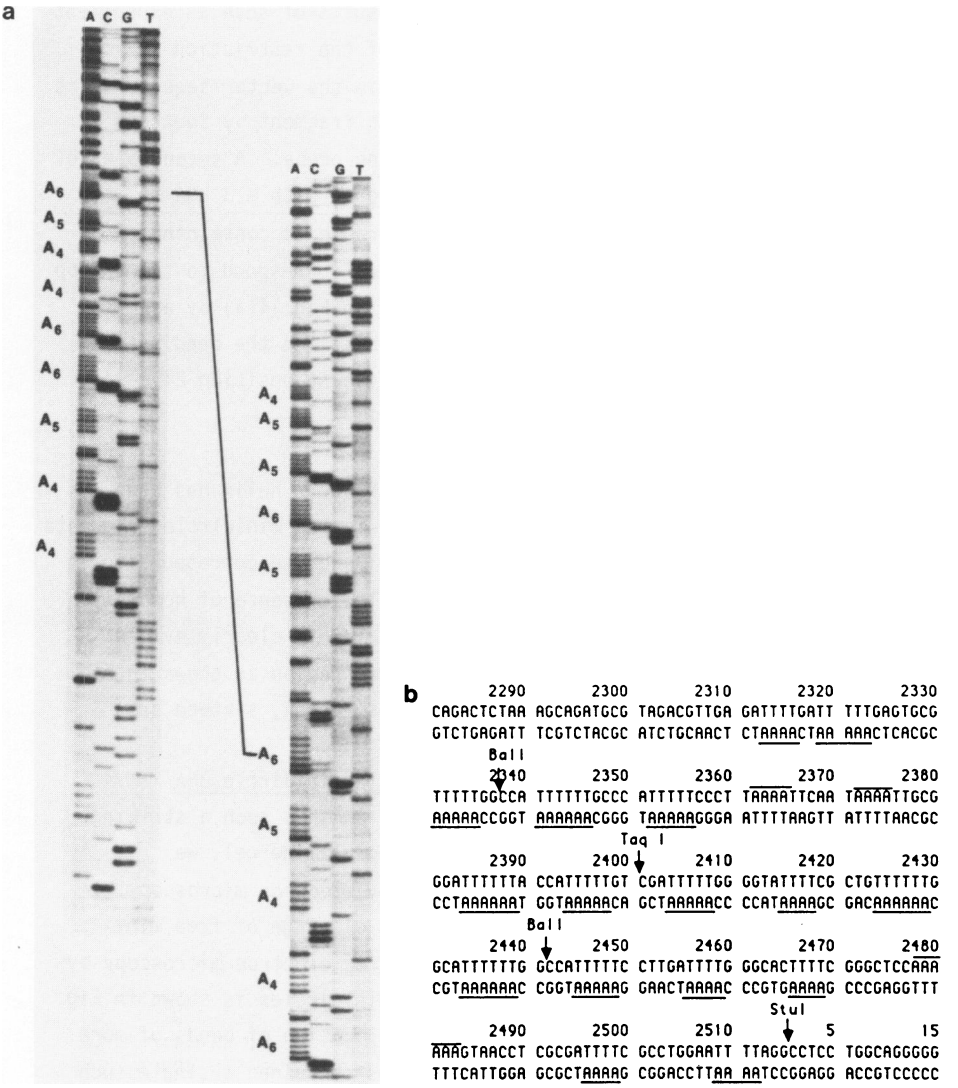

Figure 5. DNA sequence analysis of the bent helical region. (a) DNA sequencing gel showing the dideoxynucleotide chain termination sequencing of the H strand template in the counterclockwise direction from the StuI site (see Fig. 1). The resulting sequence showing tracts of 4-6 A residues is that of the L strand. The template was the single-stranded DNA of M13mp11 containing a StuI-XhoI half-length minicircle fragment. (b) The nucleotide sequence was derived from (a) and from the sequence of a fragment overlapping the StuI site (data not shown). The upper strand is the H strand and is written in the 5' to 3' direction. Oligo dA tracts in the H strand are overlined and oligo dA tracts in the L strand are underlined.

retardation due to bending of the DNA). The results of such an experiment are shown in Fig. 4(a) and (b). Because some of the restriction enzymes used to cleave the DNA also cut many times within the vector sequences, it was necessary to identify the released monomeric fragment by Southern hybridization using labeled minicircle DNA as the probe. A second blot of the gel made in a bidirectional transfer was probed with M13 DNA to identify fragments containing M13 sequences. Fragments containing minicircle sequences but lacking vector sequences correspond to the 373 bp monomeric minicircle fragment and are indicated in Fig. 4(a) by arrows. The results of this experiment shown in Fig. 4(b) place the bend between the HinfI site at position 2284 and a FnuD II site at position 2493 with the center around the TaqI site at position 2401.

Sequence of the bent helix region

The nucleotide sequence of the region of the bent helix has been determined for several clones of XhoI-StuI half-length minicircle fragments from the major sequence class. A sequencing gel and the corresponding sequence for this region are shown in Fig. 5. The presence of homopolymeric dA·dT stretches separated by 10-11 base pairs is clearly evident and confirms the localization of the bend to this region as shown in Fig. 1. Of the nineteen homopolymeric tracts in this region, sixteen are oriented in the same direction.

Electron microscopic visualization of the bend in minicircle DNA

Since the bend in the Crithidia minicircle DNA has such a striking effect on the migration of the DNA through an acrylamide gel, we anticipated that the bend might be visualized by electron microscopy. Unit-length linear minicircles were prepared by cleavage of free minicircles either with XhoI or SacII and prepared for electron microscopy by the Kleinschmidt procedure. A gallery of cut minicircles is shown in Fig. 6. Molecules were scored for the presence and location of bends of more than 90°. More than 50% of the linear molecules contained a single such bend. In the case of the XhoI-cut molecules, the bend was determined to be 1.12 kb from one end while for the SacII-cut molecules the bend was 0.41 kb from one end. The positions of the bend in XhoI- or SacII- cut molecules are consistent within the error of the measurements with the previous mapping results by electrophoretic methods and indicate that such bends can be localized directly by electron microscopic observation. Of the remaining molecules, approximately 10% showed no measurable bend while 90%

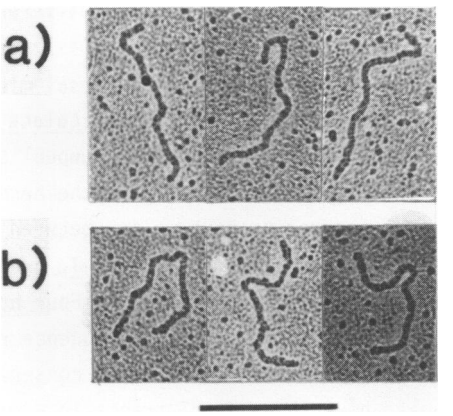

Figure 6. Electron microscopic mapping of the bent helical region. Free minicircles were isolated from purified kinetoplasts as described (18) and digested with SacII or with XhoI. After phenol extraction and ethanol precipitation each DNA sample was prepared for electron microscopy by the aqueous Kleinschmidt procedure. The relative length of each arm of all molecules having a single bend of 90° or more were measured. More than 50% of all molecules had only one such bend. (a) minicircles cleaved with SacII and (b) minicircles cleaved with XhoI. Bar indicates 0.5 microns.

contained two or more bends. In the latter case, one bend was always located at the same position as found in molecules containing only a single bend.

DISCUSSION

Koo et al., (15) and Hagerman (14) have shown that homopolymeric dA·dT tracts four to six base pairs in length and repeated in phase with the helix screw cause an intrinsic bend in DNA. We have identified a single strong bend in the major sequence class of kDNA minicircles from Crithidia fasciculata by both gel electrophoretic and electron microscopic methods. The DNA sequence in the region was shown to contain nineteen such homopolymeric tracts in a region of about 200 base pairs with sixteen of the tracts oriented in the same direction. The presence of these homopolymeric tracts with a spacing of 10-11 base pairs on the average appears to be responsible for the strong intrinsic bend in the Crithidia minicircles at this site.

The presence of only a single region of bent helix in Crithidia minicircles well separated from the two origins of replication suggests that this unusual sequence element is not an integral part of either replication

origin. This sequence organization is strikingly different from that of Leishmania tarentolae (23) in which the bent helix is located adjacent to the conserved sequence region containing the universal minicircle sequence. Although the conserved sequence regions of C. fasciculata and L. tarentolae have a high degree of homology (Sugisaki and Ray, unpublished), the homopolymeric dA•dT tracts that appear to account for the bend in Leishmania minicircles are outside of the region of homology between these species. The sequence organization of T. equiperdum (6) and T. brucei (24) minicircles is also similar to that of L. tarentolae. Four homopolymeric dA•dT tracts are contained within the conserved sequence region of these minicircles. T. lewisi minicircles which have two conserved sequence regions located 180° apart have three dA•dT tracts in a row within each conserved region with the middle tract in the opposite orientation in each case. Thus, whereas tracts of homopolymeric dA•dT are a common feature of kinetoplast minicircles, such tracts are considerably more abundant in Crithidia minicircles.

Since a single region of bent helix is found in minicircles from many trypanosome species, such structures may be involved in some biological function of minicircles in these cases. The suggestion by Marini et al. (25) that the bend may participate in the packing of the minicircles in a regular way within the network structure is an interesting possibility. Another possibility might be a role as a pilot sequence involved in guiding free minicircles to an appropriate site for their subsequent replication and/or back to the network after being replicated.

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