Volume 14 Number 13 1986 Nucleic Acids Research

Specific cleavage of kinetoplast minicircle DNA from *Leishmania tarentolae* by mung bean nuclease and identification of several additional minicircle sequence classes

Michael L.Muhich* and Larry Simpson+

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

Received 6 January 1986; Revised and Accepted 21 May 1986

ABSTRACT

Multiple sequence classes of kinetoplast minicircle DNA from Leishmania tarentolae were cleaved by mung bean nuclease in the presence of formamide, yielding unit length linear molecules which retained the anomalous electrophoretic mobility in acrylamide characteristic of minicircle DNA. No specific cleavage site sequence common to all minicircle sequence classes was apparent, although the main region of nuclease cleavage was localized approximately 350 bp from the unique SmaI restriction site of the conserved region found in all minicircle sequence classes. Covalent closure of the minicircle substrate was not a requirement for cleavage, as linearized network-derived or cloned minicircles were also cleaved by mung bean nuclease at similar locations. The partial sequences of several new minicircle sequence classes released from the network by mung bean nuclease are also reported.

INTRODUCTION

The structurally unique mitochondrial genome of the hemoflagellate protozoa, termed kinetoplast DNA (kDNA), consists of a catenated network of 5-20 X 10^3 minicircles and 20-50 maxicircles (1-3). The maxicircle represents the informational mitochondrial DNA of these cells. It consists of a conserved transcribed region encoding sequences homologous to known mitochondrial genes (4-10) and a nontranscribed region which diverges extensively in size and sequence among the various trypanosomatid genera (11-14). The function of minicircle DNA is not known. Minicircles range in size from 465 to 2300 bp, depending on the species (3); however, within any one species minicircle size is fairly uniform. A single network typically consists of multiple sequence classes of minicircles, each containing a small, 150-270 bp, region of species-specific, conserved nucleotide sequence (15, 16). Rapid rates of minicircle sequence evolution have been

© IRL Press Limited, Oxford, England.

documented in different strains of Trypanosoma brucei (17, 18) and Trypanosoma cruzi (19, 20), and in different species of Leishmania (21-23) and Crithidia (24, 25). Minicircle DNA is not transcribed in detectable amounts and sequences of cloned minicircles from T. brucei (26), T. lewisi (27) and L. tarentolae (16) reveal only short open reading frames. There is, however, a preliminary report of a possible protein product of Crithidia minicircle DNA (28), which, if confirmed and extended, would imply a genetic role for at least the large minicircles of this species.

Mung bean nuclease has been used as an endonucleolytic probe of altered DNA helix conformation for both prokaryotic and eukaryotic DNAs. The sites of mung bean nuclease single strand nicking of supercoiled PM2 phage and pBR322 DNAs have been mapped to the non-coding regulatory regions of these molecules (29-32); cleavage by mung bean nuclease occurred predominantly within A+T-rich regions and at inverted repeat sequences capable of forming hairpin structures (31, 32). Mung bean nuclease is capable of double strand cleavage of Plasmodium nuclear DNA, and possibly nuclear DNA from other lower eukaryotes, at sites before and after genes (33). Under partially denaturing conditions cleavage occurred on linear Plasmodium DNA at A+T-rich sites, which, however, were no more A+T-rich than surrounding sequences which were not cleaved by the nuclease (33).

We report here the specific double strand cleavage of multiple L. tarentolae minicircle sequence classes by mung bean nuclease. The sites of mung bean nuclease cleavage on the previously sequenced KSR1/Lt19 minicircle (16) were mapped. Cleavage site specificity was shown to be independent of the nucleotide sequence of the cleavage site and the topology of the minicircle, but was related, in those cases that could be determined, to the distance from the conserved region with the adjacent conformational "bend". We also present the partial sequences of five additional classes of minicircles released from the kinetoplast network by mung bean nuclease.

MATERIALS AND METHODS

Cell culture and DNA isolation

Cultures of L. tarentolae were grown to stationary phase in

Brain-Heart Infusion medium (Difco) supplemented with 10 ug/ml hemin at 27 C (34). Covalently closed circular kDNA was recovered as described (35) and purified by two rounds of CsCl/ethidium bromide isopycnic centrifugation. The construction and sequence of the E. coli minicircle clone pLt19 was described previously (16). Plasmid DNA was isolated as described previously (36).

Restriction enzyme and mung bean nuclease digestions

Restriction endonucleases were purchased from New England Biolabs and Bethesda Research Labs. Digestions were performed under conditions recommended by the suppliers. Mung bean nuclease was purchased from P-L Biochemicals. KDNA networks and free minicircles were digested with the indicated units of mung bean nuclease activity at a DNA concentration of 0.01 mg/ml in the reaction buffer of McCutchan et al. (33) to which 0.001% Triton X-100 was added to minimize nuclease adsorption to the plastic reaction vessel. Mung bean nuclease digestions were performed at 50 C for 30 minutes. Samples were incubated for five minutes at 50 C prior to the addition of nuclease. Reactions were terminated by phenol extraction following the addition of an equal volume of 0.02 M Tris (pH 8.0), 0.02 M EDTA, and the DNA was precipitated with ethanol.

Gel electrophoresis and DNA hybridization

Electrophoresis was carried out in submerged horizontal agarose gels and vertical polyacrylamide gels (acrylamide:bis, 29:1) in a buffer of 0.09 M Tris-borate (pH 8.3), 2 mM EDTA. DNA visualization and photography were as described (37). Southern transfer methods and in vitro labeling of DNA probes were as previously described (12). Hybridization was performed in 0.75 M NaCl, 0.075 M Na citrate, pH 7.2, 0.25% w/v nonfat dry milk (38) and 50% formamide, or 0.75 M NaCl, 0.075 M Na citrate, pH 7.2, 0.2% SDS, 0.5 mg/ml sonicated denatured salmon sperm DNA, 0.5 mg/ml poly(rA), 0.02% Ficoll, 0.02% polyvinylpyrollidine, 0.02% bovine serum albumin and 50% formamide (39) at 37° C for 12-18 hours. Hybridized filters were washed at 45 C with multiple changes of 0.015 M NaCl, 0.0015 M Na citrate, pH 7.2.

Sequence analysis of the sites of mung bean nuclease cleavage

Unit length, linearized minicircles were electroeluted from

agarose following digestion of kDNA networks with mung bean nuclease in 45% formamide, and incubated separately with BamHI, EcoRI or HindIII, which cleave various minicircle sequence classes at single sites (16). The minicircle fragments were subsequently cloned into M13 mp10 such that the termini generated by mung bean nuclease were ligated to the HincII restriction site adjacent to the annealing site for the oligonucleotide sequencing primer (40) of the M13 vector. Clones containing minicircle fragments were identified by plaque hybridization (41) and analyzed individually by agarose gel electrophoresis.

DNA sequence analysis was performed using the dideoxy-chain termination method (42), employing the modifications described previously (4). DNA sequences were analyzed with the Los Alamos Sequence Analysis System (43) and the programs of Staden (44) running on a VAX 11/780, and the Pustell programs (45) running on an IBM PC.

RESULTS

Specific cleavage of multiple sequence classes of kinetoplast minicircle DNA by mung bean nuclease

Incubation of L. tarentolae kDNA networks with mung bean nuclease in the presence of 40-55% formamide (see Materials and Methods), resulted in partial disassembly of the network complex through double strand scissions of the catenated minicircle and maxicircle DNA molecules. The results of digestions of kDNA at various mung bean nuclease/DNA ratios and at various formamide concentrations are presented in Fig. 1A. The predominant cleavage product migrated as a broad band, ranging in size from 835 to 880 bp. The size and relative abundance of this fragment was consistent with that expected for unit length linear minicircle DNA (16). Hybridization analysis (results not shown) confirmed the minicircle identity of this band and the other visible minor bands, and indicated that at a low nuclease to DNA ratio and a low formamide concentration, high molecular weight maxicircle fragments (possibly unit length; arrow, Fig. 1A) were also released from the network. Maximal release of unit length minicircle DNA from the kinetoplast network was observed at 10 units of nuclease per ug of network DNA and at a formamide

concentration of 50%. Under these conditions, a portion of the kDNA network always remained undigested and was retained at the origin of the gel.

We have previously reported that the migration of unit length minicircles and minicircle DNA fragments in acrylamide gels is retarded relative to their mobility in agarose (16). Evidence has been presented which suggests that this anomalous electrophoretic behavior is the result of the presence of a static curvature ("bend") within a specific region of the minicircle molecule (46-48). Figure 1B shows the results of electrophoresis in 5% acrylamide of unit length minicircle DNA released from network DNA by digestion with mung bean nuclease (lane 1) or BamHI (lane 2). The electrophoretic migration profile of the minicircle DNA linearized by mung bean nuclease digestion was heterogeneous and had an average, calculated size 2.1X that observed in agarose. The calculated size of the BamHI-linearized minicircle was also 2.1X that observed in agarose.

The L. tarentolae kinetoplast network contains multiple minicircle sequence classes which are distinguishable by restriction endonuclease digestion patterns (16). To examine the sequence composition of the unit length, linear minicircles released from network DNA by mung bean nuclease, aliquots of the agarose gel-isolated 835-880 bp band (Fig. 1A) were digested with several restriction enzymes. The agarose gel shown in Fig. 2 reveals that various proportions of the kinetoplast minicircle DNA linearized by mung bean nuclease digestion were sensitive to cleavage by BamHI, HindIII and EcoRI, implying a heterogeneity of mung bean nuclease-released minicircle DNA. Digestion with BamHI yielded a predominant 780 bp band and a series of minor, smaller sized fragments. HindIII cleavage resulted in the increased mobility of the forward edge of the broad, mung bean nuclease-linearized, minicircle band. This increased mobility corresponded to a calculated size of 820 bp. Incubation with EcoRI resulted in a heterogeneous mixture of minicircle digestion products, the two most prominent bands having lengths of 310 and 535 bp. On the other hand, incubation of the mung bean nuclease-generated, unit length linearized minicircle DNA with SmaI, which cuts within the conserved region of presumably all minicircle sequence

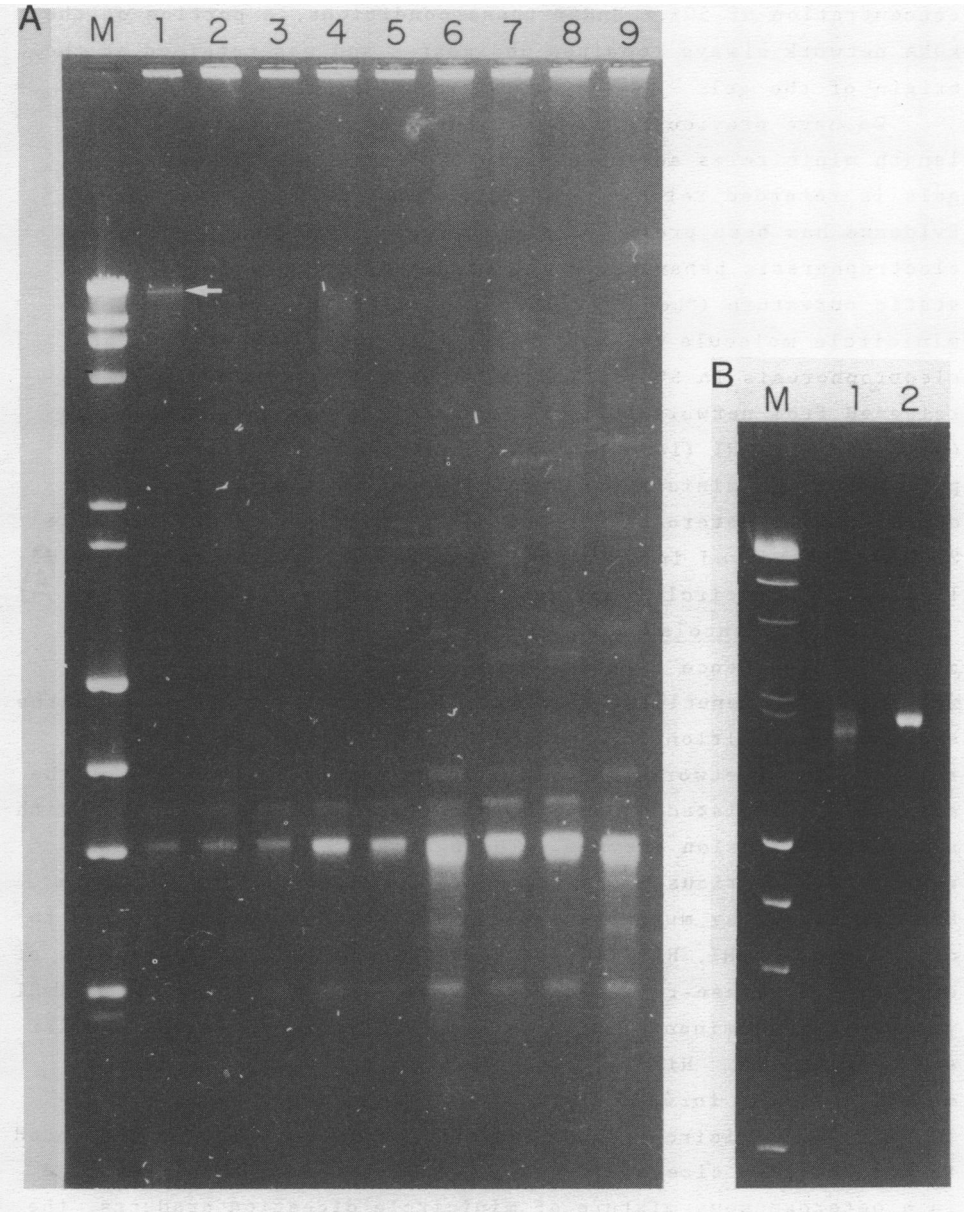


Figure 1. Release of unit length minicircles from L. tarentolae kDNA networks by digestion with mung bean nuclease. (A) Effect of varying the enzyme/DNA ratio and the concentration of formamide on the cleavage of kDNA. Approximately 1 ug of kDNA was digested with 1 (lanes 1, 2 and 3), 10 (lanes 4, 5 and 6) and 20 (lanes 7, 8 and 9) units of enzyme in the presence of 30%

(lanes 1, 4 and 7), 40% (lanes 2, 5 and 8) and 50% (lanes 3, 6 and 9) formamide, and electrophoresed through 1.2% agarose. Note: one half of the sample loaded into lane 5 (10 units mung bean nuclease/ug kDNA, 40% formamide) was lost during preparation. (B) Electrophoretic migration in 5% acrylamide of unit length minicircles, which were eluted from an agarose gel following release from the kDNA network by digestion with mung bean nuclease (lane 1) or BamHI (lane 2). M, marker DNAs: HindIII digested lambda DNA and HaeIII digested ØX174 RF DNA. The sizes, in kilobases, of the ten marker DNA bands are as follows: 23.13, 9.42, 6.56, 4.37, 2.32, 2.03, 1.35, 1.08, 0.87 and 0.60.

classes (16), resulted in apparently complete digestion and the appearance of two major bands with lengths of 345 and 500 bp. The sums of lengths of the major EcoRI and SmaI restriction products were, respectively, approximately equivalent to minicircle unit length. The appearance of discrete sized restriction products resulting from the secondary digestions implies a specificity of cleavage of minicircle network DNA by mung bean nuclease and the complete digestion by SmaI implies that the major mung bean nuclease site is located an equivalent distance from the conserved region in almost all digested minicircle sequence classes.

Nucleotide sequences surrounding the cleavage sites of mung bean nuclease on kinetoplast minicircle DNA

To define the regions of mung bean nuclease digestion on the various *L. tarentolae* minicircle sequence classes, the nucleotide sequences extending from the cleavage sites were determined for unit length minicircles released from kDNA networks by mung bean nuclease digestion and subsequently cloned into M13 after a secondary digestion with BamHI, EcoRI or HindIII (as described in Materials and Methods). The decameric sequences directly downstream of the regions of mung bean nuclease cleavage are presented in Table 1. No specific cleavage site sequence could be deduced from sequences of the mung bean nuclease generated blunt-end termini; however, in every clone examined, nuclease digestion terminated adjacent to a pyrimidine, (a thymidine in 26 out of 27 clones). In terms of the BamHI- and EcoRI-mung bean clones that are homologous to the previously described KSR1/Lt19 sequence class (16), the majority of duplex cutting occurred near nucleotide position 535 from the single EcoRI site (Fig. 3, cluster A). This places the cleavage site terminus 348 bp from

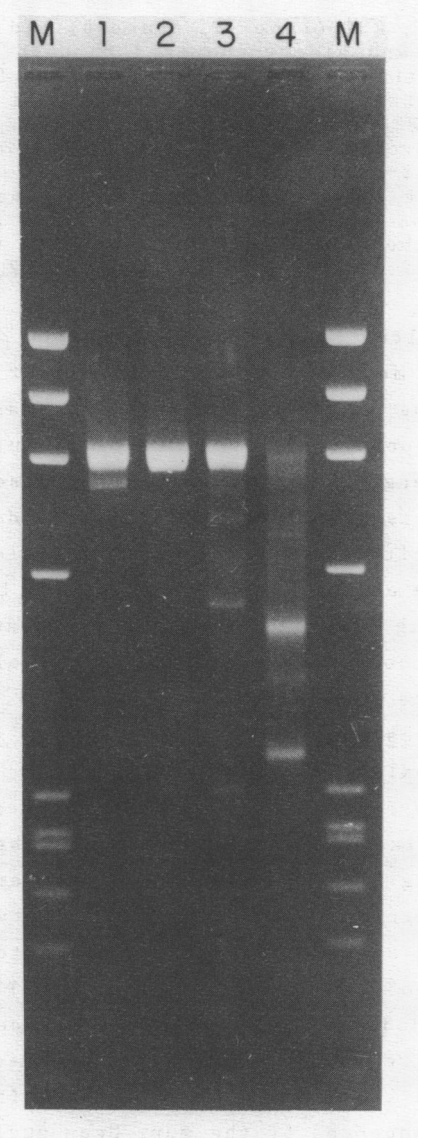


Figure 2. Restriction analysis of unit length kDNA minicircles released from networks by mung bean nuclease digestion. Approximately 0.5 ug of gel-isolated minicircle DNA was digested with BamHI (lane 1), HindIII (lane 2), EcoRI (lane 3) or SmaI (lane 4), and electrophoresed through 2.0% agarose. M, marker DNAs: HindIII digested lambda DNA and HaeIII digested ØX174 RF DNA.

Table 1. Mung bean nuclease cleavage sites on *L. tarentolae* kinetoplast minicircle DNA.

Clone[a]		Sequence[b]	pKSR1 nt coordinate[c]
B1		TGAGGGGCAT	
B2	(ATATATATTT)	TTCACTTCAA	720,721 +
B3	(TTTCCAGAAT)	TACCCCGGGG	193,194 -
B4		TAACGCTTAC	
B5	(TTTTCAATTT)	TCGTGTGAAA	142,143 +
B6	(TTACTATATA)	TGGTAATATA	550,551 -
B7	(TTAAGAGGCA)	TACCCCAGCC	518,519 -
B8		TGTATATTGA	
B10		TGAGGGGCAT	
B11		TCCAAGGCTA	
B12	(ATATAAGTTA)	TAAGGGCTTA	535,536 -
R5	(TGACATAGTA)	TAGGATCCGG	628,629 +
R6	(TATATATTTT)	TCACTTCAAC	721,722 +
R7	(TACTGTAAAT)	TAAAGATAGA	697,698 -
R8	(TTAAATAACA)	TCCAGCAAAA	751,752 -
R9	(ATATAAGTTA)	TAAGGGCTTA	535,536 -
R10	(CTATACTATG)	TCACCGCATA	621,622 -
R11	(ATTTTTCACT)	TCAACCACAT	726,727 +
R12	(TATATAGTTA)	TAAGGGCTTA	535,536 -
D2		TCCATACTGT	
D3		CGACTCTACA	
D4		TACTGTGTCG	
D5		TCCCTCAGGT	
D6		TGGTCTGTTG	
D7		TGAGGGTCGA	
D9		TACGACTCTA	
D12		TAGGGTTATA	

a. The letters B, R and D indicate the restriction enzymes, BamHI, EcoRI and HindIII, used to cleave the mung bean nuclease-linearized minicircles prior to cloning into M13.
b. Only the first 10 nucleotides downstream of the mung bean nuclease cleavage site/HincII cloning site are shown. The sequences given in parentheses are the 10 nucleotides expected to lie upstream of the cleavage site based on the individual clones homology to the pKSR1 minicircle (16).
c. The nucleotide sequences of the pKSR1 and pLT19 minicircle clones are identical except that the pLT19 sequence is numbered from the BamHI site (nucleotide position 631 on the pKSR1 sequence). The nucleotide pairs between which the nuclease cleavage sites were localized are numbered from the EcoRI site on the pKSR1 sequence, +/- indicates strand polarity relative to the pKSR1 sequence reported in ref. 16.

the SmaI restriction site and 780 bp from the BamHI restriction site on the KSR1/Lt19 minicircle; these distances agreed with the calculated fragment sizes of minicircle DNA linearized by mung

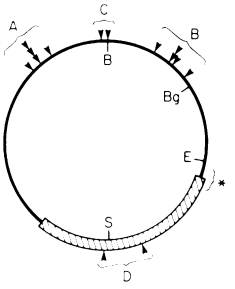


Figure 3. Diagrammatic representation of mung bean nuclease cleavage sites on the (874 bp) KSR1/Lt19 minicircle sequence class. The individual arrows correspond to the nucleotide coordinates given for the "B" and "R" clones of Table 1. (A) The cluster of nuclease cleavage sites near KSR1 nucleotide position 535/Lt19 nucleotide position 779, (B) the cluster of sites near 720/90, (C) the two sites adjacent to 631/1, (D) the two sites near 188/432. The hatched box represents the position of the conserved region (16), and the asterisk (*), the position of the bend (46, 47). B, BamHI; Bg, BglII; E, EcoRI; S, SmaI.

bean nuclease and then digested with BamHI or SmaI (Fig. 2). Five additional KSR1/Lt19 homologous clones mapped to a region surrounding nucleotide position 720 (Fig. 3, cluster B); however, these cleavage site termini were distributed over a broader range. Two cleavage site termini were located adjacent to the unique BamHI restriction site (Fig. 3, cluster C) and two within the minicircle conserved region near the unique SmaI restriction site (Fig. 3, cluster D).

Lt19 minicircle DNA cloned in E. coli is digested by mung bean nuclease in a similar manner as BamHI-linearized minicircle DNA from kDNA networks

The sensitivity of native and E. coli-cloned, BamHI-linearized, Lt19 minicircles to digestion by mung bean nuclease were compared. Both network-derived and cloned BamHI-linearized, minicircle sequences showed similar sensitivities to mung bean nuclease, and each was cleaved to yield a 780 bp digestion product (Fig. 4). This was the same sized cleavage product observed when circular, network minicircles were digested first with mung bean nuclease and then with BamHI. At the formamide concentrations and nuclease/DNA ratios employed here, only approximately 50% of the substrate DNA was cleaved by the

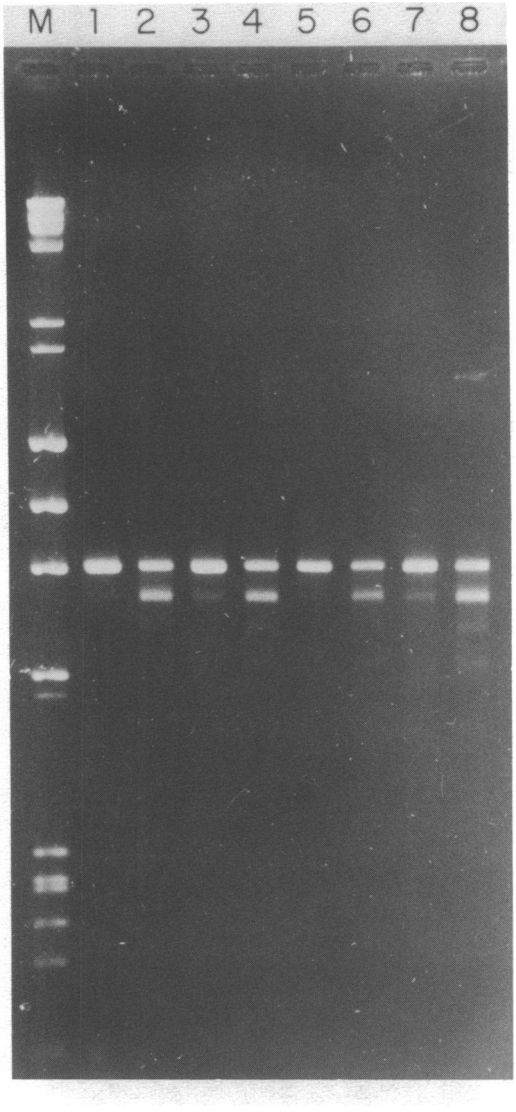
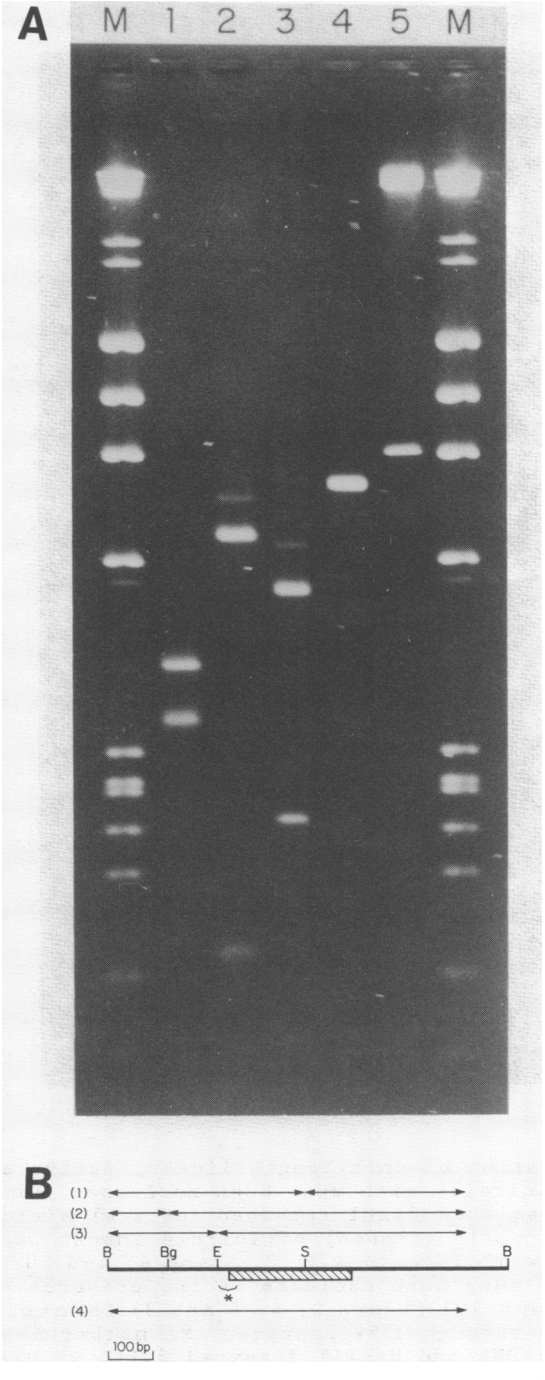


Figure 4. Digestion of unit length linear, native and cloned BamHI kDNA minicircles with mung bean nuclease. Approximately 0.5 ug of gel-isolated BamHI released (native) minicircle kDNA (lanes 1-4) and pLt19 (cloned) minicircle insert DNA (lanes 5-8) were digested with 10 (lanes 1, 2, 5 and 6) and 20 (lanes 3, 4, 7 and 8) units of mung bean nuclease in the presence of 40% (lanes 1, 3, 5 and 7) and 50% (lanes 2, 4, 6 and 8) formamide and electrophoresed through 1.5% agarose. M, marker DNAs: HindIII digested lambda DNA and HaeIII digested ϕX174 RF DNA.


A
M 1 2 3 4 5 M
B
(1)
(2)
(3)
B Bg E S B
*
(4)
100 bp

nuclease (Fig. 4, lanes 2, 4, 6 and 8). Increasing the time of incubation by a factor of two (to 60 min.) had no effect on the extent of cleavage by mung bean nuclease (results not shown).

In order to map the predominant region of mung bean nuclease cleavage at 40-50% formamide on the BamHI-excised pLt19 minicircle insert, the gel-isolated 780 bp major cleavage product was subjected to restriction analysis. Digestion of the 780 bp mung bean nuclease fragment with SmaI resulted in the production of two fragments with lengths of 348 and 418 bp, digestion with BglII yielded 135 and 652 bp fragments and digestion with EcoRI yielded a 540 bp fragment and a 240 bp fragment (Fig. 5A). These data localized the major region of mung bean nuclease cleavage on the pLt19 minicircle insert at approximately nucleotide position 780 (Fig. 5B). This corresponds almost exactly to the nucleotide position mapped by sequence analysis for the predominant cleavage site of mung bean nuclease on native KSR1/Lt19 minicircle DNA (Table 1), considering that nucleotide 535 of the KSR1 minicircle sequence corresponds to nucleotide 779 on the Lt19 minicircle sequence (16). We conclude that the major site of cleavage by mung bean nuclease at 40-50% formamide is essentially identical on both native (circular and linear) and cloned (linear) Lt19 minicircle DNA.

The results presented in Fig. 4 also demonstrate that the cleavage of unit length linearized native and cloned Lt19 minicircle DNA by mung bean nuclease is dependent to a greater extent on the formamide concentration than on the amount of nuclease activity. Moreover, faint low molecular weight bands

Figure 5. Mapping of the major mung bean cleavage site on the pLt19 minicircle DNA. (A) The predominant digestion product of mung bean nuclease treated (50% formamide) pLt19 insert (BamHI released) was gel-isolated and incubated with SmaI (lane 1), BglII (lane 2) and EcoRI (lane 3) and electrophoresed through 2.0% agarose. Lane 4 contains the gel isolated predominant digestion product of pLt19 insert DNA treated with mung bean nuclease in 50% formamide. Lane 5 contains EcoRI digested pKSR1 (unit length minicircle insert). M, marker DNAs: HindIII digested lambda DNA and HaeIII digested ϕX174 RF DNA. (B) The results are diagrammed such that the lengths of the arrowed lines are proportional to the sizes of the corresponding digestion products, numbered as in (A). The hatched box represents the position of the conserved region on the Lt19 minicircle (16), and the asterisk (*) the position of the bend (46, 47). B, BamHI; Bg, BglII; E, EcoRI; S, SmaI.

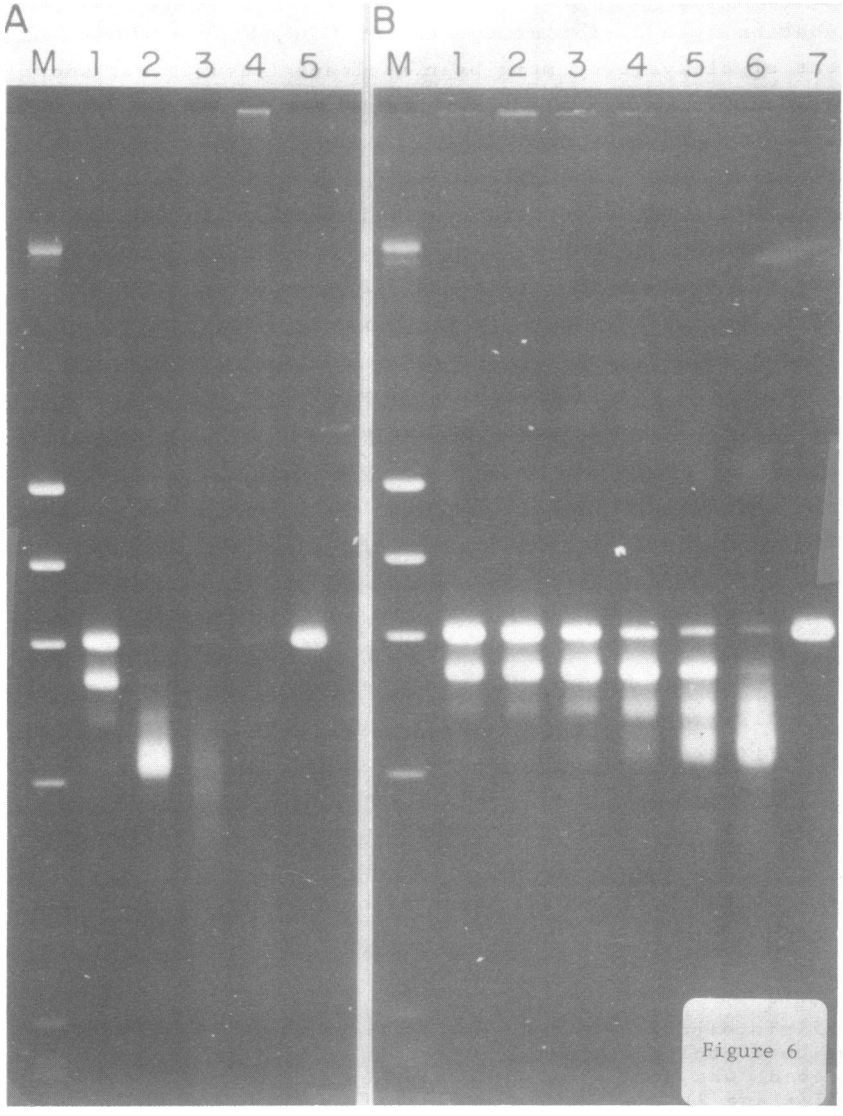


Figure 6. The effect of increasing formamide concentration on mung bean nuclease cleavage of minicircle kDNA.
(A) Approximately 0.5 ug of Lt19 insert DNA was incubated with 10 units of nuclease in the presence of 50% (lane 1), 55% (lane 2), 60% (lane 3) and 70% (lane 4) formamide and electrophoresed through 1.8% agarose. Lane 5 contains unit length Lt19 insert. M, marker DNAs: HindIII digested lambda DNA and HaeIII digested ϕX174 RF DNA. (B) Digestion of Lt19 insert with mung bean

nuclease performed as outlined in (A) except that the formamide concentration was 50% (lane 1), 51% (lane 2), 52% (lane 3), 53% (lane 4), 54% (lane 5) and 55% (lane 6). Lane 7 contains unit length Lt19 insert.

were also visible in the digests performed in 50% formamide (Fig. 4, lanes 2, 4, 6 and 8), which are the result of secondary, less frequent, mung bean nuclease cleavage events.

The effect of formamide concentration on the specificity of mung bean nuclease cleavage of unit length linear Lt19 minicircle DNA is presented in Fig. 6. The 780 bp, BamHI-mung bean nuclease fragment was the predominant product of incubation in 50% formamide (Fig. 6A, lane 1); approximately 50% of the input minicircle DNA was cleaved by the nuclease (compare with results in Fig. 4). All of the unit length Lt19 substrate DNA was cleaved at a formamide concentration of 55%, yielding a diffuse band ranging in size from 610 to 620 bp (Fig. 6A, lane 2). At formamide concentrations of 60% and 70%, the Lt19 substrate was completely degraded by the nuclease (Fig. 6A, lanes 3 and 4). Figure 6B shows that the fraction of Lt19 substrate resistant to mung bean nuclease digestion decreased as the formamide concentration was increased to 55%.

To identify the secondary regions of mung bean nuclease cleavage on the Lt19 minicircle at 55% formamide, the major digestion product (the 610-620 bp band) was gel-isolated and subjected to restriction analysis (Fig. 7A). At an agarose concentration of 2.2%, the 55% formamide/mung bean nuclease minicircle fragment migrated as a more compact band (Fig. 7A, lane 4), with an apparent length of 611 bp. EcoRI digestion of this material yielded a fragment approximately 539 bp in length and a smaller cognate fragment which has migrated off the gel. SmaI and HpaII digestion each generated a diffuse band, approximately 264 bp in length, as well as unique fragments with sizes of 347 bp and 312 bp, respectively (Fig. 7A, lanes 2 and 3). Both BamHI ends of the Lt19 substrate, at 55% formamide, were removed by mung bean nuclease digestion (Fig. 7B). One cleavage site terminus mapped to the identical location of mung nuclease action detected at 50% formamide (nucleotide position 780 on the Lt19 minicircle or 536 on the KSR1 sequence, Fig. 3A

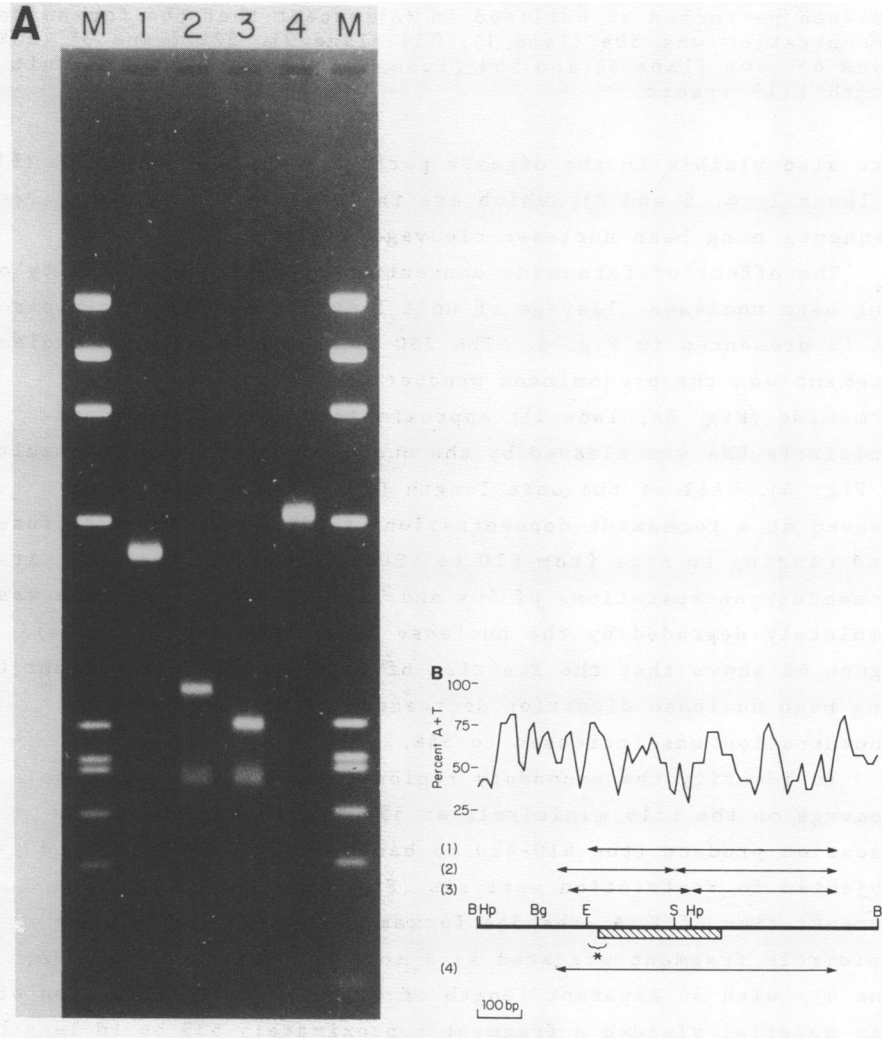


Figure 7. Mapping of the 55% formamide-mung bean nuclease cleavage sites on Lt19 minicircle DNA. (A) The predominant product of mung bean digestion of the Lt19 insert in the presence of 55% formamide was gel-isolated and digested with EcoRI (lane 1), SmaI (lane 2) and HpaII (lane 3) and electrophoresed through 2.2% agarose. Lane 4 contains the gel-isolated 55% formamide/mung bean digestion product. M, marker DNAs: HindIII digested lambda DNA and HaeIII digested ØX174 RF DNA. (B) The results are diagrammed such that the lengths of the arrowed lines are proportional to the sizes of the corresponding digestion products, numbered as in (A). The hatched box represents the position of the conserved region on the Lt19 minicircle (16) and

the asterisk (*), the position of the bend (46, 47). B, BamHI; Bg, BglII; E, EcoRI; Hp, HpaII; S, SmaI. Base composition of Lt19 plotted as percent A+T, calculated for every tenth nucleotide position using a window size of 20.

(16)). The other cleavage site terminus mapped to a region between nucleotides 169 and 204 on the Lt19 minicircle (KSR1 nucleotides 799-834). The relationship of the nuclease cleavage site termini to the A+T content of the Lt19 minicircle sequence is indicated in Fig. 7B.

Identification of several additional minicircle sequence classes from the L. tarentolae kDNA network

The partial sequences of five of the 11 BamHI-mung bean nuclease clones and all eight of the HindIII clones presented in Table 1 vary extensively from the three known L. tarentolae minicircle sequence classes (16). The partial nucleotide sequences determined from these independently-derived clones have been grouped together on the basis of sequence homology, and are presented in Fig. 8. In spite of the fact that complete sequences are not yet available, it is clear that these clones represent new minicircle sequences.

The eight HindIII clones can be separated into three non-homologous sequence "groups" (Fig. 8A, B and C). The 286 bp sequence of group A comprises five of the HindIII clones and has extensive, but interrupted, homology with the L. tarentolae Lt26 minicircle class (16). The first 135 nucleotides are homologous with a portion of the variable region of the Lt26 sequence (Fig. 9A), the next 79 nucleotides possess no significant homology with any of the known L. tarentolae minicircle sequences, and the remaining 72 nucleotides are homologous with the minicircle conserved region and have been aligned with the sequence of this portion of the Lt26 molecule (Fig. 10). The 173 nucleotide group B sequence (Fig. 8B) was compiled from two HindIII clones, D5 and D7, and is 90% homologous with a portion of the Lt26 minicircle conserved region (Fig. 9B). HindIII clone D12 comprises the group C sequence which possesses only limited homology, 30 out of 34 nucleotides, to the variable region of the KSR1/Lt19 minicircle class: nucleotides 55-89 of the group C sequence (Fig. 8C) and nucleotides 657-692 of the KSR1 minicircle (16).

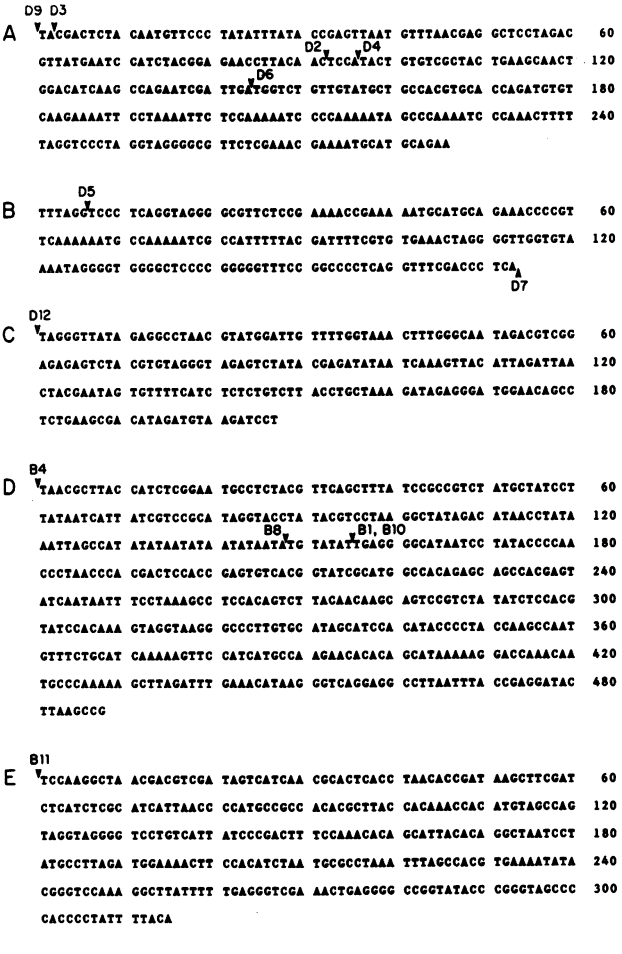


Figure 8. Partial sequences of nucleotides of several additional L. tarentolae minicircle sequence classes. The sequences were compiled on the basis of cross-homology from the independently-derived, mung bean nuclease-generated clones described in Table 1. The position of the mung bean nuclease cleavage sites on the minicircle sequences are indicated by arrows along with the corresponding clone designation. (A) the contiguous sequence obtained from mung bean nuclease-HindIII clones D2, D3, D4, D6 and D9. (B) the reverse complement of the sequence of mung bean nuclease-HindIII clone D7. This sequence encompasses the entire sequence obtained from the mung bean nuclease-HindIII clone D5. (C) the sequence obtained from the mung bean nuclease-HindIII clone D12. (D) the contiguous sequence obtained from mung bean nuclease-BamHI clones B1, B4, B8 and B10. (E) the sequence obtained from the mung bean nuclease-BamHI clone B11.

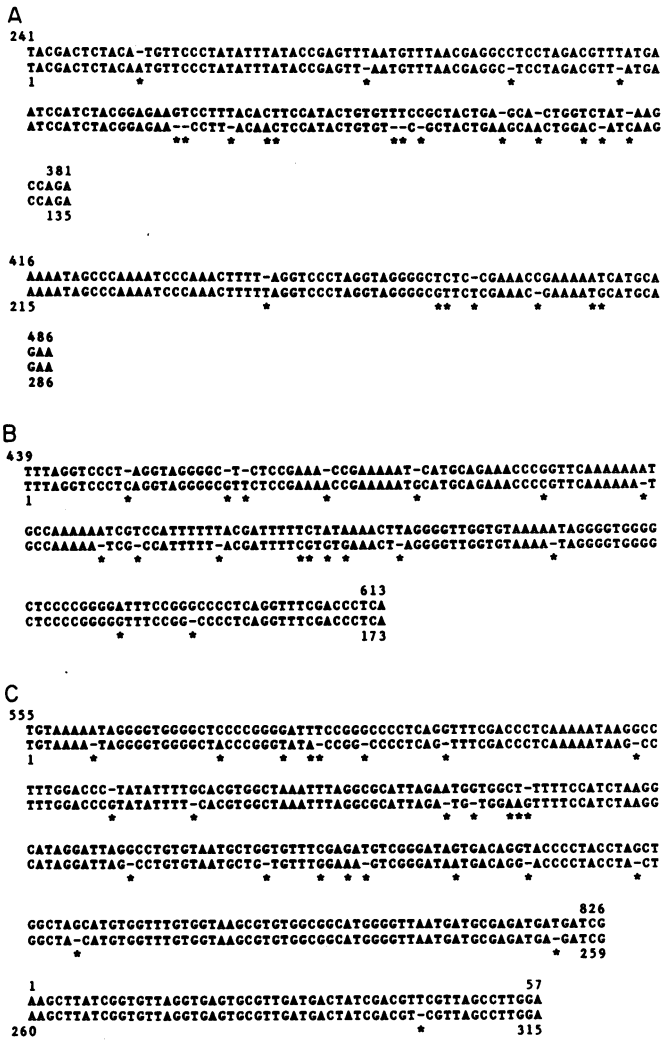


Figure 9. Locally homologous regions between the Lt26 minicircle and the minicircle sequences reported in Fig. 8A, (A); 8B, (B); 8E, (C). The non-homologous base pairs are indicated by asterisks (*) and single character pads by a dash (-). The Lt26 sequence is presented on the upper line and numbered as in Kidane et al (16). The minicircle sequences reported in this study are presented on the lower line and are numbered as in Fig. 8.

The 488 nucleotides of group D (Fig. 8D), were assembled from the sequences obtained from four of the five BamHI clones which do not map to the KSR1/Lt19 minicircle class and lack

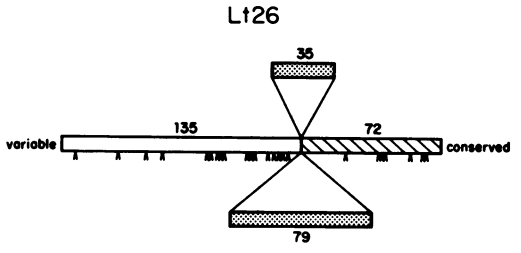


Figure 10. Diagramatic representation of the homology existing between the group A sequence and a portion of the Lt26 minicircle sequence class. The upper portion of the figure represents 242 bp of the Lt26 minicircle (nucleotides 241-381 and 416-486, ref. 16) and the lower portion of the figure the 286 bp of the group A sequence (Fig. 8A). The hatched box represents minicircle conserved region sequence, the open box represents minicircle variable region sequence, and the stippled boxes denote the nonhomologous minicircle sequences. The arrowheads correspond to the positions of point mutations within the group A sequence as compared to the Lt26 sequence.

significant homology to any of the known L. tarentolae minicircle classes. Nevertheless, BamHI clones B1, B4, B8 and B10 all yielded positive signals in plaque hybridization assays with a nick-translated kDNA probe (data not shown). Computer assisted (SEQH program from the Los Alamos sequence analysis package (43)) sequence homology searches failed to identify significant similarities between the sequences of these clones and either L. tarentolae maxicircle or M13 vector sequences. The reverse complement of the 315 nucleotide sequence obtained from the BamHI clone B11 (Fig. 8E) encompasses sequences homologous with 87 nucleotides of the Lt26 minicircle conserved region and 228 nucleotides of the Lt26 minicircle variable region (Fig. 9C). The organization of the group E sequences is similar to the Lt26 conserved/variable region junction (16); however, single base substitutions are present throughout both the conserved and variable region homologous sequences resulting in a base mismatch value of 8.25%.

DISCUSSION

Mung bean nuclease has been shown to cleave at small, single stranded gaps in duplex DNA (29), and to nick supercoiled DNAs at

A+T-rich regions (31) and at inverted repeats capable of forming hairpin structures (31, 32). In addition, mung bean nuclease has been shown to produce double strand breaks before and after genes in Plasmodium (33) DNA in the presence of 30-45% formamide. The recognition mechanism for this gene-releasing activity is unknown, but probably is a function of the conformation of the extragenic regions of Plasmodium DNA.

We have shown in this paper that kDNA minicircles are linearized by mung bean nuclease in the presence of 40-50% formamide. The analysis of this phenomenon is complicated by the presence of an undefined, but small number of minicircle sequence classes in network DNA. We attempted to simplify the analysis by examining in detail those minicircle classes, the complete sequences of which are already known- the EcoRI, BamHI KSRI/Lt19 class, and the two HindIII classes, Lt26 and Lt154. This was the rationale for the subcloning procedure involving digestion of the mung bean nuclease-released, linear, minicircle DNA with BamHI, EcoRI or HindIII prior to cloning into M13 for sequence analysis. The partial sequences of 14 of the 27 clones examined were almost identical with the KSRI/Lt19 sequence, allowing, together with information from restriction mapping, a precise identification of the major region of nuclease cleavage.

Covalent closure of the minicircle substrate was not a requirement for digestion, as linearized and circular, network Lt19 minicircles and cloned Lt19, linear insert DNA were cleaved at an identical location in 40-50% formamide. Nine additional minicircle clones mapped to three other regions of the KSRI/Lt19 sequence.

The main conclusion from both the partial sequencing and the restriction mapping studies is that the major region of cleavage by mung bean nuclease is localized at an approximately equivalent distance from the conserved region in all digested minicircle sequence classes although there is no conserved consensus sequence for nuclease recognition. While this is a relatively A+T-rich region in the Lt19 minicircle, it is not the region of highest local A+T content. We speculate that the mung bean enzyme may be recognizing a specific transition in DNA conformation induced by the partial denaturing conditions. We further speculate that such a transition may be a function of some

unusual structural feature of the minicircle molecule that is preserved in the linearized state, such as the static bend located at one side of the conserved region (16, 47). The recognition of this induced conformational state by mung bean nuclease is not a generalized feature of all single-strand specific nucleases, since S1 nuclease under identical reaction conditions did not cleave minicircle DNA in a similar fashion (results not shown). The structure, if any, being recognized by the mung bean nuclease is unknown.

The formamide concentration had a pronounced effect on the extent and specificity of cleavage of unit length, linear Lt19 DNA. Raising the formamide concentration above 50% led to the appearance of a second major region of nuclease cleavage, the terminus of which was located between nucleotides 169 and 204 on the Lt19 minicircle. The apparent variation in the extent of mung bean nuclease cleavage at 55% formamide could result from either the clustering of multiple nuclease cleavage sites or variability in the DNA structure recognized by the nuclease or from different degrees of duplex nibbling of this terminus by the nuclease.

In conclusion, further analysis of the cleavage of kDNA minicircles by mung bean nuclease may lead to a better understanding of the mechanism of recognition of structural features in duplex DNA by this endonucleolytic probe.

An offshoot of this study was the discovery of several additional classes of minicircle sequences from the L. tarentolae kDNA network. The sequences of 13 of the 27 analyzed mung bean nuclease-derived minicircle clones varied extensively from the known sequences of the KSRI, Lt26 and Lt154 minicircle classes (16). The partial sequences obtained were assembled into five separate minicircle groups. Two of the minicircle sequence groups, the 207 nucleotide group C sequence and the 488 nucleotide group D sequence, possess only limited homology to any of the three known L. tarentolae minicircle sequences. The fact that all five of the clones which comprise these two sequence groups hybridize with kDNA and lack detectable homology with maxicircle sequences indicates that the group C and D sequences are of minicircle origin.

The 286 nucleotide group A sequence consists of a stretch of 79 nucleotides possessing no significant homology to any of the three known minicircle classes juxtaposed between a 135 nucleotide sequence which is homologous with the Lt26 variable region and 72 nucleotides homologous to the minicircle conserved region. This sequence arrangement is consistent with that expected for the product of a recombinational exchange occurring between the Lt26 sequence class and another undescribed minicircle sequence class, or it may represent the involvement of the Lt26 minicircle in (at least two) independent deletion and insertion events.

The possibility of cloning artifacts giving rise to this type of sequence rearrangement can never be entirely eliminated. However there was no evidence of instability of cloned minicircle sequences in the rec A- E. coli host used for these experiments.

The mechanism responsible for the rapid rate of minicircle sequence evolution is not known. Chen and Donelson (26) suggested from sequence analysis of two T. brucei minicircles that extensive recombinational exchange between different minicircle sequence classes could account for the extensive heterogeneity observed in the minicircle population of T. brucei. In the case of the large minicircles from Crithidia, indirect evidence in the form of restriction analyses and electron microscopy of heteroduplexes indicated that the minicircles evolve mainly through segmental rearrangements (24, 25). The two- and four-fold symmetrically organized minicircle conserved region repeats of, respectively, T. lewisi (27) and T. cruzi (49) may also represent the product of minicircle recombinational events. The organization of the "group A" minicircle sequences from L. tarentolae is also consistent with a recombinational exchange mechanism.

Single base changes, in the form of substitutions, deletions or insertions, also contribute to the overall rate of minicircle sequence variation. This is evident from the degenerative homology shared between segments of the Lt26 minicircle class and the group A, B and E minicircle sequences. Point mutations were found to be distributed in an overall uniform fashion across both the conserved and variable regions of the minicircle fragments

analyzed here. In contrast, the sequences of the EcoRI and BamHI clones mapping to the KSR1/Lt19 sequence class contained few, if any, base changes, in agreement with previous results on the homogeneity of this sequence class (16).

ACKNOWLEDGEMENTS

This work was supported in part by grant AI-09102 from the National Institutes of Health to L.S. M.L.M. was the recipient of a U.S. Public Health Service National Research Service Award, GM-07104 and a Dr. Ursula Mandel Scholarship from UCLA.

* Current address: Department of Medical Microbiology, Stanford University School of Medicine, Stanford CA 94305

+ To whom reprint requests should be addressed

REFERENCES

1. Simpson, L. (1972) Intern. Rev. Cytol. 32, 139-207.
2. Borst, P. and Hoeijmakers, J. (1979) Plasmid 2, 20-40.
3. Simpson, L. (1985) Intern. Rev. Cytol. In press.
4. de la Cruz, V., Neckelmann, N. and Simpson, L. (1984) J. Biol.Chem. 259, 15136-15147.
5. de la Cruz, V.F., Lake, J.A., Simpson, A.M. and Simpson, L. (1985) Proc. Natl. Acad. Sci. USA 82, 1401-1405.
6. de la Cruz, V., Simpson, A., Lake, J. and Simpson, L. (1985) Nucl. Acids Res. 13, 2337-2355.
7. Benne, R., DeVries, B., Van den Burg, J. and Klaver, B. (1983) Nucl. Acids Res. 11, 6925-6941.
8. Hensgens, A., Brakenhoff, J., De Vries, B., Sloof, P., Tromp, M., Van Boom, J. and Benne, R. (1984) Nucl. Acids Res. 12, 7327-7344.
9. Payne, M., Rothwell, V., Jasmer, D., Feagin, J. and Stuart, K. (1985) Mol. Biochem. Parasitol. 15, 159-170.
10. Sloof, P., Van den Burg, J., Voogd, A., Benne, R., Agostinelli, M., Borst, P., Gutell, R. and Noller, H. (1985) Nucl. Acids Res. 13, 4171-4190.
11. Borst, P., Fase-Fowler, F., Hoeijmakers, J. and Frasch, A. (1980) Biochim. Biophys. Acta. 610, 197-210.
12. Muhich, M., Simpson, L. and Simpson, A.M. (1983) Proc. Natl. Acad. Sci. USA 80, 4060-4064.
13. Muhich, M. and Simpson, L. (1985) Nucl. Acids Res. 13, 3241-3260.
14. Maslov, D., Kolesnikov, A. and Zaitseva, G. (1984) Mol. Biochem. Parasitol. 12, 351-364.
15. Donelson, J., Majiwa, F. and Williams, R. (1979) Plasmid 2, 572-588.
16. Kidane, G., Hughes, D. and Simpson, L. (1984) Gene 27, 265-277.
17. Steinert, M., Van Assel, S., Borst, P. and Newton, B.A. (1976) In The Genetic Function of Mitochondrial DNA,

(Saccone, C. and Kroon, A.M., eds), Elsevier/North-Holland, Amsterdam, 71-81.
18. Borst, P., Fairlamb, A.H., Fase-Fowler, F., Hoeijmakers, J.H.J. and Weislogel, P.O. (1976) In The Genetic Function of Mitochondrial DNA, (Saccone, C. and Kroon, A.M., eds.), 59-69.
19. Morel, C., Chiari, E., Camargo, E., Mattei, D., Romanha, A. and Simpson, L. (1980) Proc. Natl. Acad. Sci. USA 77, 6810-6815.
20. Macina, R.A., Sanchez, D.O., Affranchino, J.L., Engel, J.C. and Frasch, A.C.C. (1985) Mol. Biochem. Parasitol. 16, 61-74.
21. Simpson, L., Simpson, A., Kidane, G., Livingston, L. and Spithill, T. (1980) Am. J. Trop. Med. Hyg.,Suppl. 29, 1053-1063.
22. Arnot, D. and Barker, D. (1981) Mol. Biochem. Parasitol. 3, 47-56.
23. Wirth, D. and Pratt, D. (1982) Proc. Natl. Acad. Sci. USA 79, 6999-7003.
24. Hoeijmakers, J. and Borst, P. (1982b) Plasmid 7, 210-220.
25. Hoeijmakers, J., Weijers, P., Brakenhoff, C. and Borst, P. (1982) Plasmid 7, 221-229.
26. Chen, K. and Donelson, J. (1980) Proc. Natl. Acad. Sci. USA 77, 2445-2449.
27. Ponzi, M., Birago, C. and Battaglia, P.A. (1984) Mol. Biochem. Parasitol. 13, 111-119.
28. Shlomai, J. and Zadok, A. (1984) Nucl. Acids Res. 12, 8017-8028.
29. Kroeker, W.D. and Kowalski, D. (1978) Biochemistry 17, 3236-3243.
30. Kowalski, D. (1984) Nucl. Acids Res. 12, 7071-7086.
31. Sheflin, L.G. and Kowalski, D. (1984) Nucl. Acids Res. 12, 7087-7104.
32. Sheflin, L.G. and Kowalski, D. (1985) Nucl. Acids Res. 13, 6137-6154.
33. McCutchan, T.F., Hansen, J.L., Dame, J.B. and Mullins, J.A. (1984) Science 225, 625-628.
34. Simpson, L. and Braly, P. (1970) J. Protozool. 17, 511-517.
35. Simpson, A.M. and Simpson, L. (1980) Mol. Biochem. Parasitol. 2, 93-108.
36. Hughes, D., Simpson, L., Kayne, P. and Neckelmann, N. (1984) Mol. Biochem. Parasitol. 13, 263-275.
37. Brunk, C. and Simpson, L. (1977) Anal. Biochem. 82, 455-462.
38. Johnson, P.A., Gautsch, J.W., Sportman, J.R. and Elder, J.H. (1984) Gene Anal. Techn. 1, 3-8.
39. Simpson, L., Simpson, A. and Livingston, L. (1982) Mol. Biochem. Parasitol. 6, 237-257.
40. Messing, J. and Vieira, J. (1982) Gene 19, 269-276.
41. Benton, W.D. and Davis, R.W. (1977) Science 196, 180-182.
42. Sanger, F. Nicklen, S. and Coulsen, A. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
43. Kanehisa, M.I. (1982) Nucl. Acids Res. 10, 183-196.
44. Staden, R. (1982) Nucl. Acids Res. 10, 2951-2961.
45. Pustell, J. and Kafatos, F.C. (1984) Nucl. Acids Res. 12, 643-655.

46. Wu, H. and Crothers, D. (1984) Nature 308, 509-513.
47. Hagerman, P. J. (1984) Proc. Natl. Acad. Sci. USA 81, 4632-4636.
48. Marini, J.C., Effron, P.N., Goodman, T.C., Singleton, C.K., Wells, R.D., Wartell, R.M. and Englund, P.T. (1984) J. Biol. Chem. 259, 8974-9.
49. Leon, W., Frank, A., Hoeijmakers, J., Fase-Fowler, F., Borst, P., Brunel, F. and Davison, J. (1980) Biochim. Biophys. Acta 607, 221-231.