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Analysis of sequences of two different classes of Kinetoplast DNA minicircles of a *Leishmania* Spp.†

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Abstract. We have determined the nucleotide sequences of the minicircles representing a major (pLURkE3) and a minor (pLURkH13) class populations from the kinetoplast DNA of *Leishmania* strain UR6. These minicircles have sequence organization similar to other kinetoplastid parasites, however, they have some unique structural features. These features include the following: (i) imperfect inverted repeat in the variable regions, similar to the conserved sequence elements of guide RNA genes in African trypanosomes, (ii) tandem and non-tandem direct repeats of 8 bp or longer scattered throughout the minicircles, (iii) non uniform strand distribution of bases throughout the minicircles and (iv) high TG content, more than half of the molecules being extremely (T + G) versus (A + C) strand biased.

The heterogeneity of minicircle sequences in the variable regions may be exploited in developing recombinant DNA based diagnostic probes for detection and classification of Leishmania species.

Keywords. *Leishmania* Spp.; kinetoplast DNA; minicircle; DNA sequences.

1. Introduction

Kinetoplast DNA (kDNA) of protozoan parasites exists as a massive network structure composed of thousands of topologically interlocked, double stranded covalently closed circular DNA molecules of two kinds. Maxicircles representative of conventional mitochondrial DNA, are 20–30 kb in size with 50–100 copies in a network. It is the minicircle component made up of 10000 or so intercatenated 0·5–2·7 kb long, DNA circles of unknown functions, which is responsible for the unusual characteristics of kDNA (Borst and Hoeij makers 1979; Simpson 1986). Heterologous DNA sequences and their rapid rate of evolution imply a role other than the conventional protein coding ascribed to these minicircles (Chen and Donelson 1980).

Reports of minicircle transcription in *Crithidia* (Fouts and Wolstelholme 1979) and more recently in *Trypanosoma brucei* (Rohrer *et al* 1987) revive the question of whether minicircles do indeed have a genetic function.

The sequences of minicircles from several trypanosomatid species have been

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reported (Barrois *et al* 1981; Frasch *et al* 1981; Jasmer and Stuart 1986a; Macina *et al* 1986). A common feature is the presence of a conserved sequence region of 100–300 bp (Barrois *et al* 1981). Outside this conserved region the minicircles have only limited homology and this region is termed as variable region. In *Leishmania tarentolae* and in *T. brucei* the variable regions of the minicircles have been found to be involved in encoding guide RNAs for editing maxicircles mRNAs (Pollard *et al* 1990; Sturm and Simpson 1990).

The protozoan parasites of the genus *Leishmania* present a complex set of clinical features ranging from benign cutaneous infections to lethal systemic infections. Therefore, detailed investigations leading to characterization and identification of the organisms causing different disease states is of utmost importance in understanding and combating the disease.

Therefore in order to have a better understanding of this organism and to investigate the possibility of developing recombinant DNA-based diagnostic probes, we have studied the structural organization of the minicircles in the kDNA network. We report here the sequences of two minicircles belonging to two different sequence classes of *Leishmania* strain UR6 and the interesting features unique to these minicircles are discussed.

2. Materials and methods

2.1 *Materials*

Restriction *enzymes*, T4 DNA ligase, DNA polymerase 1 (Klenow enzyme), pancreatic RNase were purchased from Bethesda Research Laboratories (USA), ^{32}P-labelled deoxyribonucleotides were purchased from Amersham (UK). All other chemicals were of reagent grade.

2.2 *Parasite culture and kDNA purification*

Leishmania strain UR6 (MHOM/IN/1978/UR6) was originally isolated from the bone-marrow of a patient admitted to the Calcutta School of Tropical Medicine. The patient had hepatosplenomegaly and was diagnosed as a Kala-azar patient on the basis of classical clinical manifestations and identification of parasites on a bone-marrow smear. Since then UR6 promastigotes have been maintained in Ray's modified medium (1932) and subcultures were made at 72 h intervals. This strain has an isozyme pattern similar to *L. tropica* as tested in the laboratories of Professor J A Rioux, University of Paris, France and Dr R Killick-Kendrick of Imperial College, London. The method of kDNA isolation and purification has been described previously (Dasgupta *et al* 1986).

2.3 *Cloning and sequencing of full length kDNA minicircles*

The plasmid pGEM4Z has been used to clone unit length minicircles (830 bp) linearized by *Eco*RI and *Hind*III from the kDNA of *Leishmania* Spp. strain UR6 promastigotes using standard techniques (Sambrook *et al* 1989). Two such recom-

binants pLURkE3 and pLURkH13 were selected for large scale plasmid preparation and subsequent DNA sequence determination. pLURkE3 is a plasmid that contain *Eco*RI released minicircle which represent a major sequence class population and pLURkH13 is a plasmid containing *Hind*III linearized minicircle and representing a minor sequence class population of minicircles in the kDNA network. The recombinant plasmids are stable and do not get deleted on prolonged subcultures. The DNA sequencing followed the dideoxy chain termination method of Sanger *et al* (1977).

2.4 *Computer analysis*

The minicircle sequences were analysed and compared with other minicircle sequences using a software program DNASIS which uses a modification of Needleman–Wunsch (1970) algorithm and secondary structures were predicted using the modification of Pipas and McMahon (1975) algorithm.

3. Results

We have obtained an estimate of the frequency distribution of the various minicircle sequence classes in the kDNA network of *Leishmania* strain UR6 by restriction enzyme digestion and quantitative gel electrophoresis. There is a clear evidence for a predominant sequence class which comprises 45% of the network and several minor minicircle sequence classes (Dasgupta and Majumder 1989). Two minicircles representing a major and a minor sequence population in the network were cloned and sequenced. The sequences of the minicircles are shown as depicted schematically in figure 1. Analysis of sequences demonstrates that these minicircles contain a region of homology of approximately 80% which could be located within 190 nucleotides in both the minicircles (figure 1). For comparison, the sequences are aligned at a common site beginning at the conserved sequence. The conserved region has been found to be present in almost all the kinetoplastid parasites (Nasir *et al* 1987). The sequence of pLURkE3 and pLURkH13 minicircles have been compared with the cloned minicircle sequences from *L. mexicana* (Rogers and Wirth 1988) and *L. tarentolae* (Kidane *et al* 1984). The minicircles in pLURkE3 and pLURkH13 display considerable homology with other minicircles within 50 bp of the conserved region (figure 1). The conserved region contains the 13 nucleotide sequence 5′-GGGGTTGGTGTAA-3’ termed the universal minicircle sequence or UMS which has been found to be perfectly homologous in all the minicircles that have been sequenced to date. This 13 bp UMS is the origin of replication (Ryan and Englund 1989a, b; Tzfati *et al* 1992) and is present at the 3′-end of the conserved region of both the minicircles. A second UMS like sequence 5’-GGGGTTTCTGTAT-3′ is present in the opposite orientation at a distance of 50 nucleotide 5′-upstream of 13 bp UMS. The UMS is located 84 bp downstream from another highly conserved sequence element 5’-GGGCGT-3’. This sequence element which appears to control the initiation of synthesis of first Okazaki fragment (Ryan *et al* 1988) is absent in the 5’-upstream region of UMS like sequence.

An interesting feature chracteristic to these *Leishmania* minicircles is the presence of several tandem and nontandem direct repeat sequences of 8 bp or longer scattered

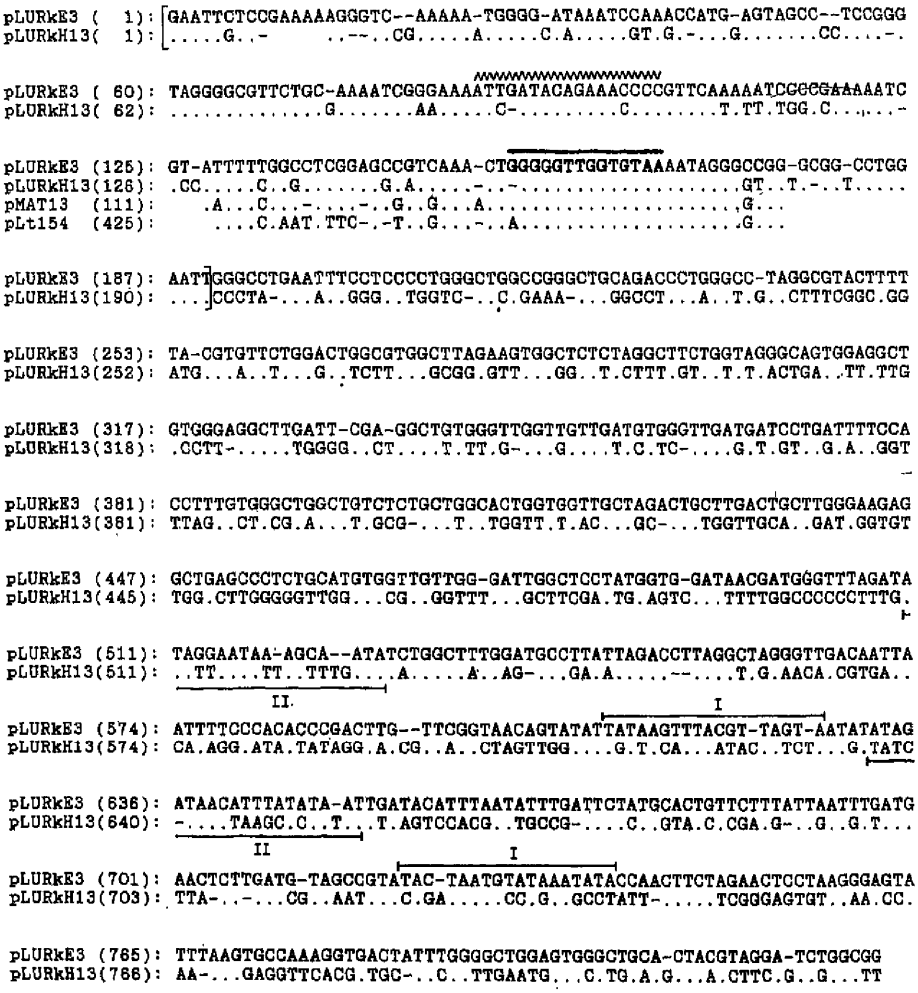

Figure 1. Comparison of nucleotide sequences pLURkE3 (top) and pLURkH13 (bottom). pLURkE3 sequence begins at a unique *Eco*RI site. pLURkH13 sequence is positioned underneath pLURkE3 so that their respective 13-mers are aligned. Dots (.) indicate positions of nucleotide identity, dashes (-) show deletions introduced to maximize alignment. The box indicates the conserved region. The UMS is marked with a thick bar and the complementary strand of UMS like sequence by a wavy line. The 18-mer imperfect inverted repeat in pLURkE3 and 20-mer imperfect inverted repeat in pLURkH13 are indicated by I and II respectively. pMAT13 and pLtl54 are cloned minicircles from *L. mexicana* amazonensis (Rogers and Wirth 1988) and *L. tarentolae* (Kidane *et al* 1984).

throughout the minicircles as shown in table 1. To find out the statistical significance of these repeats we have generated sequences each of length 1 kb, which are random but have base compositions and with similar dinucleotide distributions frequency as that of pLURkE3 or pLURkH13. These sequences were then searched for the direct repeats listed in table 1. Among 30 sequences generated in this way, only one showed a direct repeat of 'ACTGCTTG'. None of the other sequences listed in table 1 has been found to be repeated in any of these 30 sequences. The

Table 1. Search for tandem and nontandem direct repeats of 8 bp or longer was made in the whole molecule of pLURkE3 and pLURkH13.

	Position	Sequence
(A) Direct repeats in pLURkE3 minicircle		
Tandem repeats	309	GGAGGCTGTG
		******* **
(a)	319	GGAGGCTTTGG
		******* **
(b)	330	GGAGGCTGTG
	423	ACTGCTTG
		********
	431	ACTGCTTG
Non-tandem repeats	337	TG-TGGG-TTGG
		** **** ***
(a)	354	TG-TGGG-TTGA
		** **** **
	384	TG-TGGG-CTGG
		** *** **
	410	TGGTGGT--TGC
		** *** **
	461	TG-TGG--TTGT
		** *** ***
	467	TGTTGGGATTGG
		* **** ** *
	495	CGATGGGTTTAG
(b)	5	TCCGAAAAA--GGG
		** ***** ***
	17	TC--AAAAAT-GGG
		** **** ***
	69	TCTGCAAAATCGGG
		** ******
	104	TC--AAAAATCCCC
		* *******
	114	CCCGAAAAATCGTA
		* ***** *
(c)	209	TGGGCTGGC
		*********
	386	TGGGCTGGC
		********
	787	TGGGCTGGA
		*******
	798	TGGGCTG-C
(d)	84	AATTGATACA
		**********
	648	AATTGATACA
(B) Direct repeats in pLURkH13 minicircle		
Non-tandem repeats	193	TCCCTAG-AAATTGGGCCT
		*** * * *** *******
(a)	213	TCC-TCGGAAACTGGGCCT
(b)	8	CGAAAAAGT
		******* *
	18	CGAAAAAAT
		******* *
	82	CGAAAAA-T
(c)	307	TGATGTT-GTTGGCCTTG
		******* ***** ***
	362	TGAGGTT-GTTGGTATTG
		*** * ***** ***
	435	TGATGGGTGTTGGGCTTG
		* * * * ***** ***
	597	T-AGGCTAGTTGGTATTG

Nucleotide position correspond to their locations in the strand shown in figure 1. Asterisks (*) represents a match and a dash (-) represents a space inserted for alignment of the sequences.

sequences 'AATAGACA' and 'GGAGGCTGTG' have not even occurred for once in any of these sequences. This is not surprising because the probability of occurrence of these sequences of 10 bases in sequences having same base composition and dinucleotide distribution as pLURkE3 is of the order of 10^{-6} *(i.e.* 1 in 10^6 bases). This shows that the occurrence of direct tandem and nontandem repeats in sequences pLURkE3 and pLURkH13 are significant from statistical point of view. Although the minicircles contain repeat sequences, they are stable as recombinant plasmids and do not get deleted in subsequent passages. Figure 2 shows the Harr-plot analysis of pLURkE3 versus pLURkE3 itself, pLURkH13 versus pLURkH13 itself and

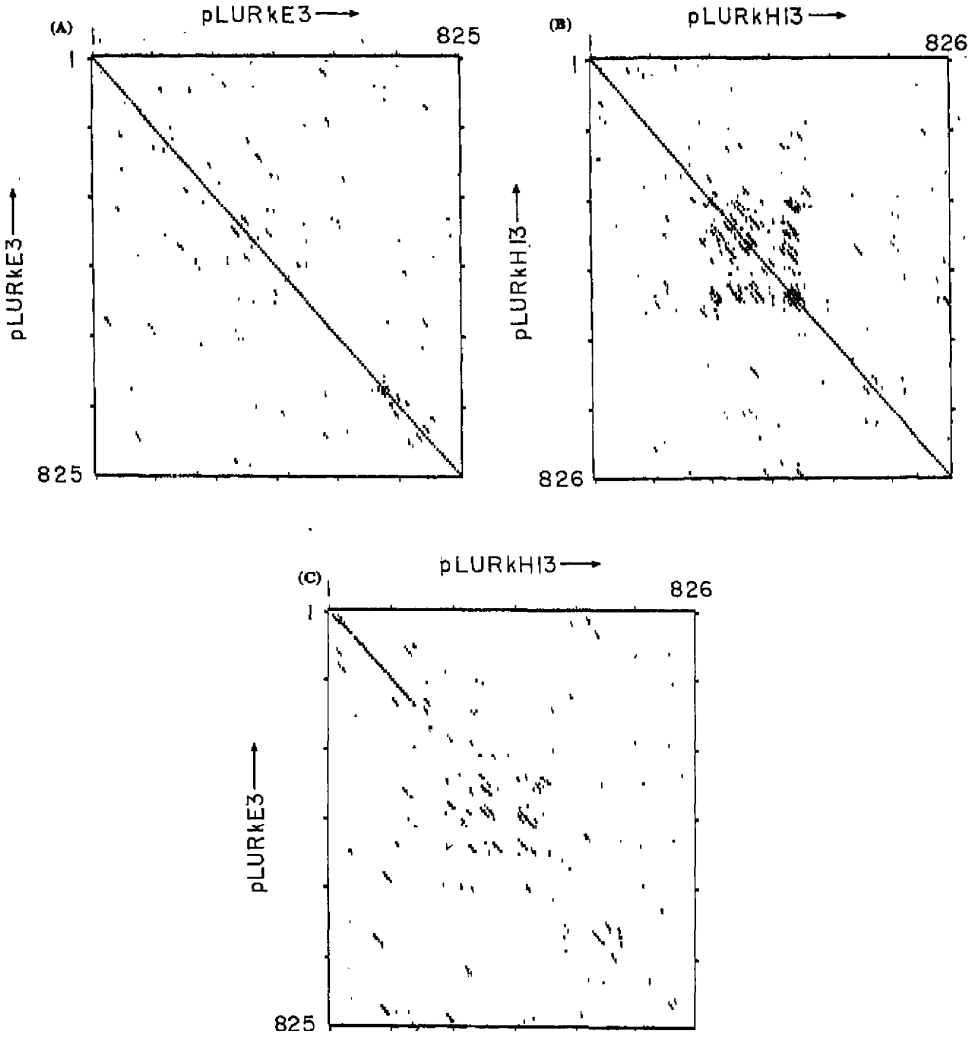

Figure 2. Harr-plot analysis of *Leishmania* strain UR6 minicircles. **(A)** Internal repeats in the minicircles in pLURkE3 by dot matrix graphics. A window of 30 nucleotides was used with a 'proportional' match of 17/30. **(B)** Internal repeats in the minicircles in pLURkH13. Same conditions as for **(A). (C)** Harr-plot analysis of pLURkH13 (horizontal) versus pLURkE3 minicircle sequences (vertical). Same conditions as for **(A).**

pLURkE3 versus pLURkH13. We have used a relatively low stringency (17/30 proportional algorithm). In case of pLURkE3 versus pLURkE3 it is clear that there is no organized sequence reiteration but random distribution of small repeat sequences of 8 bp or longer in the minicircle pLURkE3 (figure 2A). The same is true for pLURkH13 which also contains small repeat sequences randomly distributed throughout the minicircle (figure 2B), The functions of the small repeat sequences are yet to be determined, Comparison of pLURkE3 and pLURkH13 at the above stringency (17/30 proportional algorithm) shows a straight line running at 45 degree indicating the homology of the two minicircles (figure 2C) located in the conserved regions.

The AT content of pLURkE3 and pLURkH13 are both 54% which is much lower than that observed in African trypanosomes (Barrois *et al* 1981; Jasmer and Stuart 1986a). The TG content of pLURkE3 and pLURkH13 are 58% and 62% respectively. The important feature of these two minicircles is the nonuniform strand distribution of bases in different parts of the molecules as evidenced from an analysis of the local variations of T plus G and A plus C nucleotides along each strand of two minicircles (figure 3). The distribution of AT sequences in the conserved region of pLURkE3 and pLURkH13 are 52% and 53% whereas in the variable regions are 54% and 53% respectively, As can be seen in the figure 4 the region representing approximately 80% of the minicircle length in pLURkH13 (positions 125–826) and 51% of the minicircle length in pLURkE3 (positions 125–550) are extremely (T + G) versus (A + C) strand biased.

Using conventional genetic code it was found that several short open reading frames are present in both the minicircles. The longest possible polypeptide encoded by these regions are 86 and 44 amino acids in pLURkE3 and pLURkH13 respectively and in their complementary strands 57 and 34 amino acids respectively. The open reading frames are present in the variable regions of the minicircles.

The most interesting feature characteristic to these minicircles is the presence of 18 and 20 bp imperfect inverted repeat sequences flanking 89 and 109 nucleotides in their variable regions of pLURkE3 and pLURkH13 (figure 1). These 18 and 20 bp imperfect inverted repeats are 5′-TATAAGTTTACGTTAGTA-(89 bp)-TACTAA-TGTATAAATATA-3′ in pLURkE3 and 5′-ATATTAATATTAGTTTGATA-(109 bp)-TATCTAACTAAGCTCTATAT-3′ in pLURkH13 and are present only once in the minicircles.

A distinctive feature of minicircle DNA from most species of kinetoplastidae is the presence of a sharp bend in the DNA causing it to migrate anomalously slowly in Polyacrylamide gels (Kidane *et al* 1984). DNA sequence analysis suggests that the bend is the consequence of runs of 4–6 adenine residues separated by 10–11 bp or one turn of the DNA helix (Koo *et al* 1986). We observed the same phenomenon in most minicircles in different degrees in UR6 kDNA, released by digestion with various enzymes in unit length or fragmented form. This can be seen in figure 4 which shows the circular map of major class minicircles. The construction of restriction map was complicated by the fact that some of the minicircle fragments exhibited an abnormal electrophoretic mobility in acrylamide gels. The molecular weights derived from electrophoresis in agarose gels were therefore used for map construction. The fragments that migrate anomalously in acryilamide are indicated by "abnormal migration coefficients" *(e.g.* 1·26x or 1·4x). The abnormal migration coefficient is the ratio of apparent molecular weight from migration in 5% Polyacrylamide *vs* migration in 1· 5% agarose (Kidane *et al* 1984).

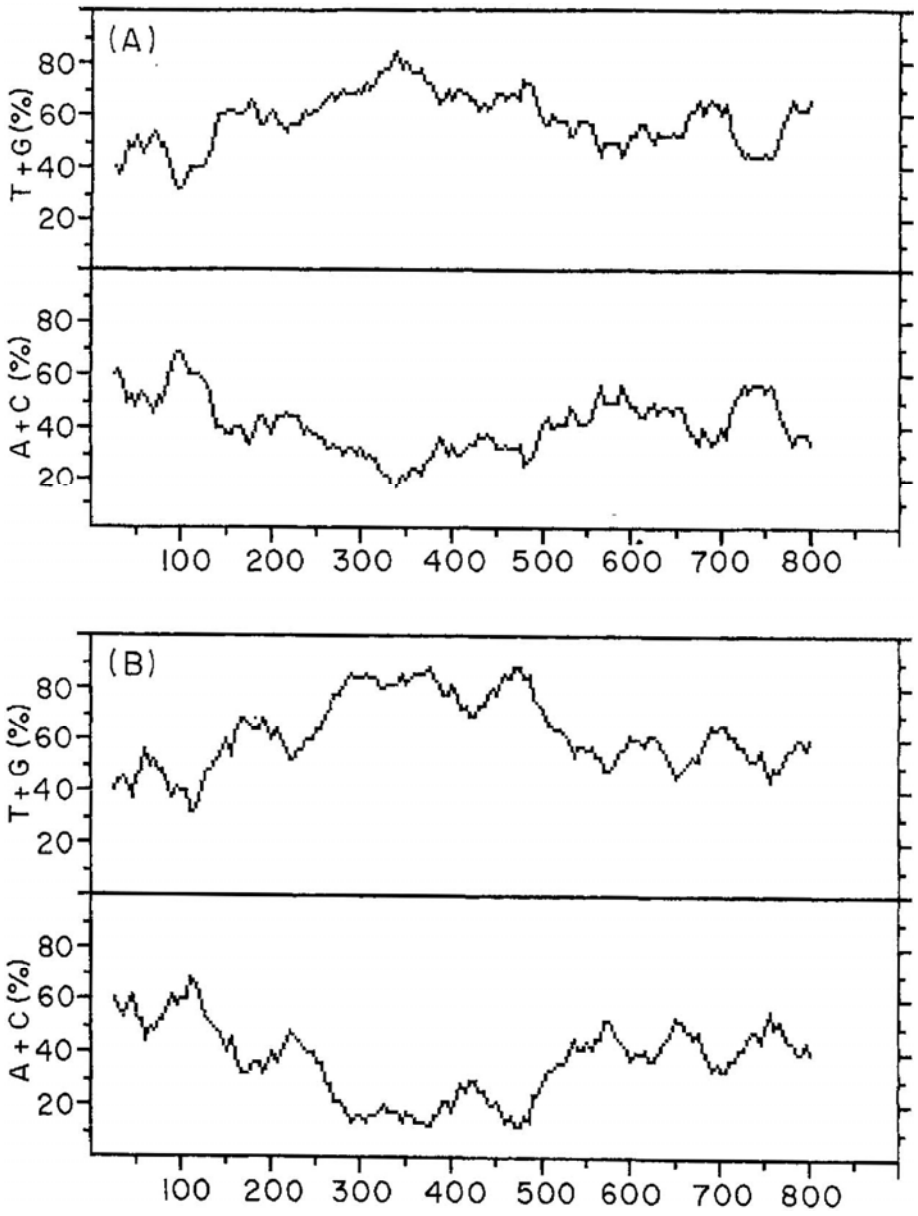

Figure 3. Local variation of T + G and A + C nucleotides of *Leishmania* strain UR6 minicircles in pLURkE3 **(A)** and pLURkH13 **(B).** The percentage of the sum of two nucleotides in a window of 50 bases moving with a step of one base was plotted versus the distance.

*Eco*RI cleaved minicircle fragments displayed less abnormal migration. Sequence analysis of minicircles in pLURkE3 and pLURkH13 show that they contain runs of A's and T's that are periodically spaced (figure 1) and these regions coincided with those detected as containing the conserved sequences.

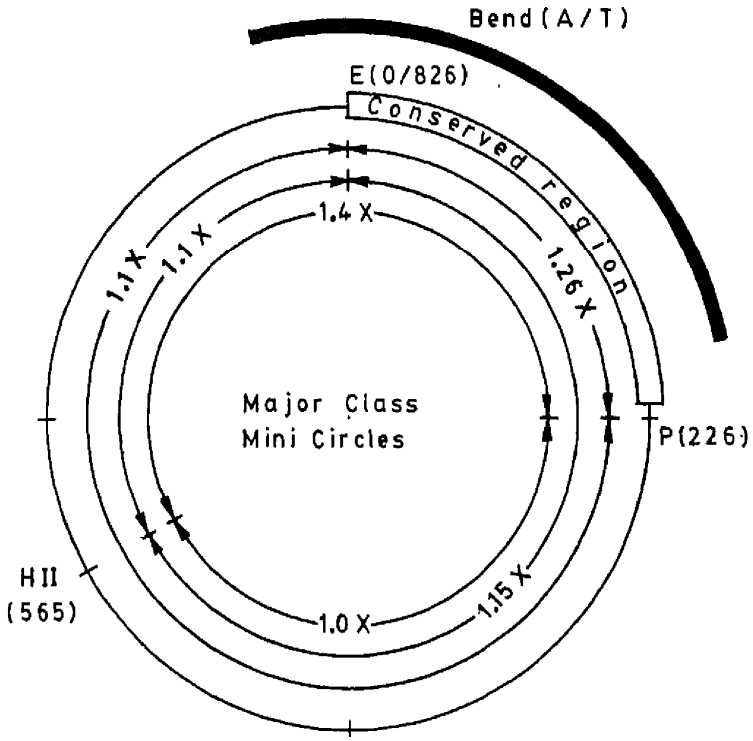

Figure 4. Restriction map of the major class minicircle. Circular map was constructed on the basis of molecular weights derived from electrophoresis in 1·5% agarose gel. The "abnormal migration coefficients" are indicated in each fragment (e.g. 1·26x or 1·4x). E, *Eco*RI; P, *Pst*I; HII, *Hinc*II.

4. Discussion

The extent of sequence heterogeneity is a species dependent property of kinetoplastidae (Kleisen *et al* 1976). The number of minicircle sequence populations in a network are known to vary from highly heterogeneous in *T. brucei* to completely homogenous in *T. equiperdum* (Steinert and Van Assel 1980). *Leishmania* strain UR6 kDNA contains a predominant sequence class comprising 45 % of the network and several minor minicircle sequence classes. An approach to understanding minicircle function is the comparative analysis of their structural organization. The determination of the sequences of two minicircles belonging to different sequence classes has shown that these molecules are organized similarly into a conserved region and a variable region. Both of them contain the universal 13-mer motif (5''GGGTTGGTGTAA-3′) termed the UMS and is located at the 3′-end of the conserved region. Extensive studies have been carried out on free minicircle replication intermediates of trypanosomes and it has been shown that minicircles with a newly synthesized L-strand initiates replication from this region (Ryan and Englund 1989a, b; Sheline *et al* 1989; Tzfati *et al* 1992). One of the minicircles contain an 18 bp and the other a 20 bp imperfect inverted repeat sequences in their variable regions. The 18 bp imperfect inverted repeat sequences were also found in the variable regions of the

minicircles in *T. brucei, T. equiperdam* (Jasmer and Stuart 1986b) and in *T evansi* (Ou *et al* 1991) but not in *L mexicana* amazonensis (Rogers and Wirth 1987) and *L tarentolae* (Sturm and Simpson 1991). It has been shown that the region flanked by 18 bp imperfect inverted repeat sequences in the variable regions contain genes for guide RNAs (gRNA) in African trypanosomes (Pollard *et al* 1990; Sturm and Simpson 1990) *L tarentolae* minicircles also encode gRNAs in the variable region, but the conserved sequence elements for gRNA genes in African trypanosomes are not present in *L. tarentolae* (Sturm and Simpson 1991). The significance of these imperfect inverted repeat sequences in *Leishmania* strain UR6 minicircles remains unclear. However, the imperfect inverted repeat sequences present in the variable regions of these minicircles mimic insertion sequences flanking mobile genetic elements in other organisms (Jasmer and Stuart 1986a).

The conformational bend described in the minicircles of most of the species of kinetoplastidae is also present in these two minicircles of *Leishmania* strain UR6 and these bent helical structures are located within the conserved regions. Our results corroborate studies by previous workers who have observed that the "DNA bending locus" of minicircles in some species of kinetoplastidae is found within or near the conserved sequence (Barrois *et al* 1981; Sturm and Simpson 1991). Interestingly, minicircles in *L. mexicana amazonensis* do not contain stretches of A's and no anomalously migrating fragments were detected in the minicircles (Rogers and Wirth 1987). On the other hand, minicircles in *L. tarentolae* exhibit very high abnormal mobilities in Polyacrylamide gels (Kidane *et al* 1984).

DNA sequences that diverge at different rates within the same kDNA network represent potentially valuable tools in diagnostic, epidemiological and ecological studies of *Leishmania.* Through the use of recombinant DNA technology, probes having species, isolate subspecies or strain specificities are being constructed and used in hybridization studies to distinguish between organisms for solving the problems of taxonomy (Rogers and Wirth 1987; Lawrie *et al* 1985). It was shown that the variable region of a minor class minicircle in this *Leishmania* strain UR6 represents a potentially valuable tool in the taxonomic identification of *Leishmania* strains (Dasgupta *et al* 1991). Following sequence analysis of these minicircles synthetic oligonucleotides may be constructed for use in polymerase chain reaction (PCR) and a rapid and sensitive method can be developed for detection of *Leishmania* parasites in clinical infections (Rogers and Wirth 1988; Smyth *et al* 1992; de Bruijn and Barker 1992; Bhattacharya *et al* 1993).

In conclusion, our analysis shows that *Leishmania* strain UR6 minicircles have common structural organization like in other *Leishmania* species, but they have some unique features. These features include the presence of tandem and nontandem direct repeats throughout the minicircles, an imperfect inverted repeat in the variable regions identical to conserve sequence elements for gRNA genes and non-uniform strand distribution of bases throughout the minicircles. It is obvious that functional studies are necessary to understand the roles of these sequence elements in the minicircles of this *Leishmania* Spp.

Acknowledgements

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