The EMBO Journal Vol.17 No.3 pp.838–846, 1998

Pankinetoplast DNA structure in a primitive bodonid flagellate, *Cryptobia helicis*

Julius Lukeš[1,2], Milan Jirků, Nuraly Avliyakulov and Oldřich Benada[3]

Institute of Parasitology, Czech Academy of Sciences and [1]Faculty of Biology, University of South Bohemia, Branišovská 31, 37005 České Budějovice and [3]Institute of Microbiology, Czech Academy of Sciences, Vídeňská 1083, 110 00 Prague 10, Czech Republic

[2]Corresponding author
e-mail: jula@paru.cas.cz

The mitochondrial DNA (mtDNA) of a primitive kinetoplastid flagellate *Cryptobia helicis* is composed of 4.2 kb minicircles and 43 kb maxicircles. 85% and 6% of the minicircles are in the form of supercoiled (SC) and relaxed (OC) monomers, respectively. The remaining minicircles (9%) constitute catenated oligomers composed of both the SC and OC molecules. Minicircles contain bent helix and sequences homologous to the minicircle conserved sequence blocks. Maxicircles encode typical mitochondrial genes and are not catenated. The mtDNA, which we describe with the term 'pankinetoplast DNA', is spread throughout the mitochondrial lumen, where it is associated with multiple electron-lucent loci. There are ~8400 minicircles per pankinetoplast-mitochondrion, with the pan-kDNA representing ~36% of the total cellular DNA. Based on the similarity of the *C.helicis* minicircles to plasmids, we present a theory on the formation of the kDNA network.

Keywords: catenation/kDNA/kinetoplastida/mitochondrial DNA/supercoiling

Introduction

Evidence gathered so far from various mitochondrial genomes favors the theory that all present day mitochondria are descendants of a single early endosymbiotic event (Gray and Spencer, 1996), although a polyphyletic origin of the mitochondrion cannot be excluded (Simpson and Thiemann, 1995). In all organisms studied, circular or linear mitochondrial DNA contains varying numbers of genes with a slow but steady tendency of mitochondrial genes to be transferred into the nucleus over the course of evolution (Palmer, 1997). For unknown reasons, however, the mitochondrial (kinetoplast) DNA (kDNA) of kinetoplastid protozoa retained the most complicated DNA structure known in nature.

In an insect trypanosomatid *Crithidia fasciculata*, kDNA represents ~15% of the total cellular DNA and is composed of ~5000 minicircles and 25 maxicircles, topologically interlocked in a single network which is located in the mitochondrial matrix close to the kinetosome of a single flagellum (Robinson and Gull, 1994; Shapiro and Englund, 1995). The kDNA network forms a disc of 1 μm in diameter and 0.3 μm thick (Ferguson *et al.*, 1992) with an extremely high DNA concentration (Rauch *et al.*, 1993). Recently it has been shown that each minicircle is connected to three neighbors by a single interlock (Chen *et al.*, 1995a), the number of interlocks increasing during the kinetoplast division up to six per minicircle (Chen *et al.*, 1995b). Furthermore, minicircles are relaxed rather than negatively supercoiled because the supercoiling would probably be incompatible with the sophisticated structure of the network (Rauch *et al.*, 1993).

Maxicircles are homologs of the mitochondrial DNA of other eukaryotes. They bear mitochondrial genes coding for ribosomal 9S and 12S RNAs and subunits of the respiratory chain complexes. Several transcripts undergo unusual processing by uridilate insertions and deletions, called RNA editing. This is mediated by guide RNAs which are encoded on maxicircles and minicircles (reviewed by Simpson and Thiemann, 1995; Arts and Benne, 1996).

Most information available on the kinetoplastid mitochondrion has been obtained from studies on three model organisms—*Trypanosoma brucei, Leishmania tarentolae* and *C.fasciculata*, all belonging to the obligatory parasitic and medically important suborder Trypanosomatina. Although ubiquitous, and thus ecologically significant, members of the second suborder Bodonina, which comprises free-living bodonids, as well as commensalic and parasitic cryptobiids, have largely been neglected. Based on morphology, the latter suborder was considered more primitive (Vickerman, 1976), this notion being recently confirmed by phylogenetic studies using various nuclear (Fernandes *et al.*, 1993; Maslov *et al.*, 1994; Wiemer *et al.*, 1995; Lukeš *et al.*, 1997) and mitochondrial (Lukeš *et al.*, 1994) genes. As an early branch of the kinetoplastid phylogenetic tree, bodonids represent objects of choice for studies of the evolution of several unusual processes known to occur in flagellates (Cavalier-Smith, 1997).

Bodonids and cryptobiids have already proved useful for studies of the evolution of RNA editing. In the fish parasite *Trypanoplasma borreli*, and recently in the free-living *Bodo saltans*, a unique gene order, editing patterns and guide RNAs have been described, thus supporting the 'editing ancient' scenario (Lukeš *et al.*, 1994; Maslov and Simpson, 1994; Yasuhira and Simpson, 1996; D.Blom, A.de Haan, M.Van den Berg, P.Sloof, M.Jirků, J.Lukeš, R.Benne, in preparation). Similarly, studies of bodonids may shed light on the origin and evolution of catenated kDNA network. Early studies of *Bodo caudatus* kDNA revealed its non-interlocked structure (Hajduk *et al.*, 1986), while a more detailed analysis of the *T.borreli* kDNA revealed large circular and linear molecules, probably composed of concatenated minicircles (Yasuhira and Simpson, 1996; Simpson, 1997). However, in these species, the kDNA appears to be so different, both from each other

and from the classical kDNA network, that no feasible evolutionary theory can be drawn.

During the ultrastructural study of *Cryptobia vaginalis* from leeches, Vickerman (1997) noted that its kDNA seemed to be dispersed throughout the mitochondrial matrix and coined for such an arrangement the term 'pankinetoplast'. Further ultrastructural analyses of selected bodonids revealed that the pankinetoplast morphology is widespread among members of the suborder Bodonina (Brugerolle *et al.*, 1979). However, any information about this enigmatic structure has been missing until now. In this work, we show that the pankinetoplast of *Cryptobia helicis* contains a uniquely organized mitochondrial DNA, with both relaxed and supercoiled minicircles, which remain largely noncatenated. We term this kind of DNA 'pan-kinetoplast DNA' (pan-kDNA). We also present a theory that attempts to explain the origin of catenated kDNA networks.

Results

Topology of the pan-kDNA

Because the standard protocols failed to isolate kDNA from *C.helicis*, we isolated total cellular DNA and subjected it to agarose gel electrophoresis (Figure 1A). Unexpectedly, a large amount of undigested DNA entered the gel; the two most prominent bands migrated at 2.6 and 4.4 kb, or 4.2 and 7.0 kb when compared with linear or supercoiled markers, respectively. Most DNA migrated in the compression zone and only a small amount of DNA remained in the slot. When total DNA of another bodonid, *T.borreli*, was analyzed under the same conditions, bands of this type were not observed. Furthermore, in the case of catenated kDNA of *C.fasciculata,* only a low intensity band of linear 2.4 kb minicircles could be detected (Figure 1A, lanes 3 and 4).

To further investigate the topology of the dominant bands visible in *C.helicis* total DNA, it was treated with topoisomerase II and subjected to agarose gel electrophoresis, followed by Southern blotting using a cloned *C.helicis* minicircle Z4 (see below) (Figure 1B). Relaxation of the fast migrating band (2.4 kb) into a population of species with varying linking number and open circles was observed, indicating that original molecules were supercoiled. Treatment with topoisomerase I, DNase I or Fe^{2+} ions, resulted in the conversion of the fast migrating band into open circles migrating at 4.4 kb. Treatment with Fe^{2+} ions produced a band at 4.2 kb which represents linear molecules. Significant mobility changes also occurred in several higher molecular weight bands which are present in the untreated sample. We assume that the weak band visible only in undigested DNA, slightly above the most prominent open circle band, is a dimer composed of two singly interlocked supercoiled minicircles (SC–SC) (Figure 1B). It is the first species to disappear after any treatment. The dimer composed of open and supercoiled rings (OC–SC) is only slightly more stable and withstands short incubations with topoisomerases. On the contrary, the dimer containing two interlocked open circles (OC–OC) becomes the most abundant species after the treatment with topoisomerase I, since it transforms the available dimers into this species by nicking (Figure 1B). In the agarose gels, it is difficult to distinguish between individual

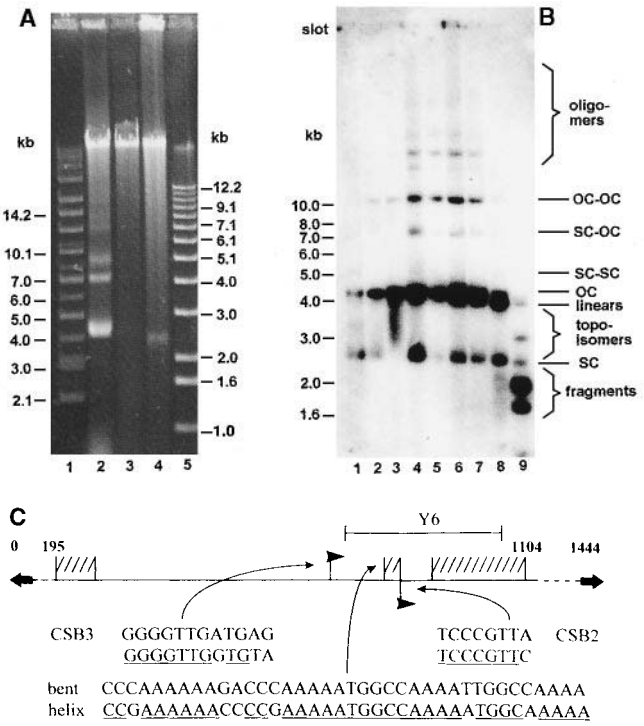

Fig. 1. Topology of the pan-kDNA. (**A**) Agarose gel electrophoresis of undigested DNA of selected kinetoplastids. DNA was detected by UV after ethidium bromide staining. Supercoiled ladder (lane 1); *C.helicis* (lane 2); *T.borreli* (lane 3); *C.fasciculata* (lane 4); linear 1kb ladder (lane 5). (**B**) Southern blot analysis of the topology of *C.helicis* minicircles. The size of the molecular weight marker (linear 1kb ladder) is shown. Agarose gel electrophoresis of DNA was performed in the absence of ethidium bromide. The slots were filled in by agarose and the gel was exposed to 600 kJ at 254 nm to nick the circles before blotting and the blot was hybridized with the minicircle Z4 probe. Untreated (lane 1) and treated DNA (lanes 2–9); 4U topoisomerase II at 30°C overnight or for 60 min (lanes 2 and 3); 25U topoisomerase I at 37°C for 30 min or 180 min (lanes 4 and 5); 400 ng DNA + 15 mM Na_2HPO_4 + 75 mM NaCl + 10 mM $FeSO_4$ + 0.15% H_2O_2 at 25°C for 1 min; stopped by addition of thiourea to concentration 100 mM (lane 6); 400 ng DNA + 4 mM $MgCl_2$ + 27 mM Tris, pH 7.5 + 1.5 mM $CaCl_2$ + 2.5U DNase I at 4°C for 1 h (lane 7). Restriction digestion with 5U of *Bgl*II or *Mva*II at 37°C for 1 h (lanes 8 and 9). (**C**) Map of the 1444 bp Z4 minicircle fragment. The bent helix-like regions are hatched. The CSB2 and CSB3 motifs, and part of the conserved bent helix of *C.helicis* minicircle (upper lane) are compared with those of *C.fasciculata* minicircle. Identical nucleotides are underlined; the Y6 clone is indicated.

types of catenanes composed of three or more interlocked circles, and in the pan-kDNA there are not enough of them for the analysis of a gel-isolated DNA by electron microscopy. However, since topoisomerase I nicks all the supercoils, we assume that the prominent band migrating at 15.0 kb is an open circle trimer (OC–OC–OC) (Figure 1B). Although >95% of the Z4 minicircle signal hybridized with DNA in the gel, the possibility that some larger catenanes remained in the slot cannot be excluded. In lanes 8 and 9 (Figure 1B), the restriction digestion of total DNA by *Bgl*II, which linearized most of the minicircles and *Mva*II, which cuts minicircles into small fragments, is visible.

The *Bgl*II-linearized minicircles were isolated from the gel, several 4.2 kb inserts were cloned, and the clone Z4 was analyzed further. To investigate a possible homology of the Z4 circle with minicircles, the bent helix region of

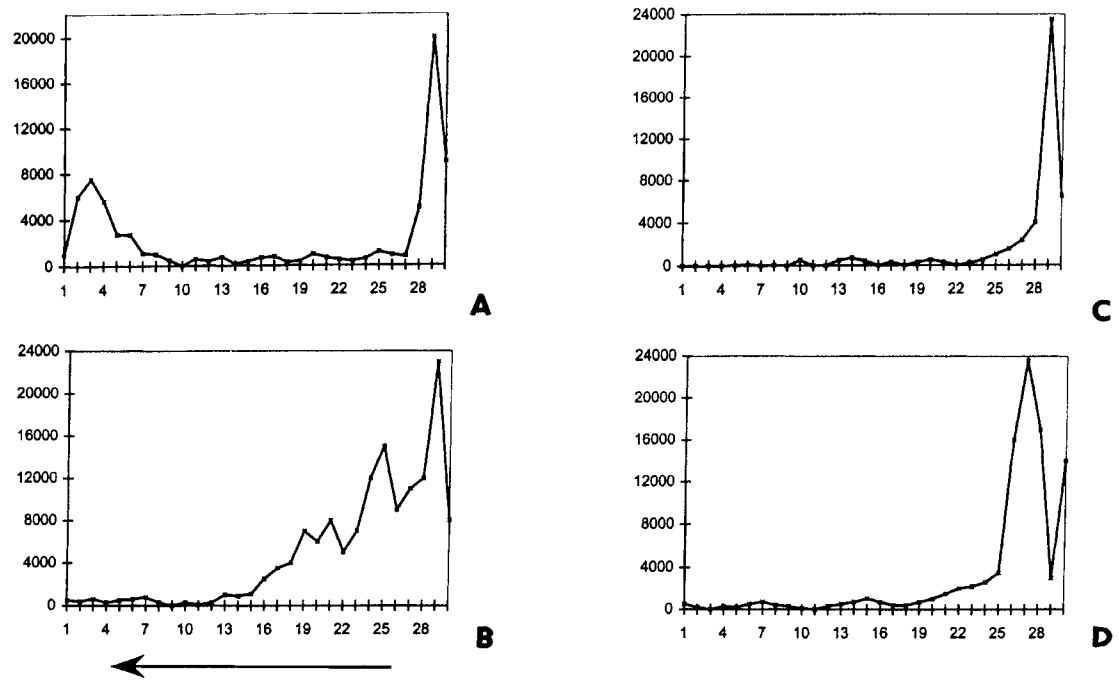

Fig. 2. Sucrose gradient centrifugation. x-Fraction No.; y-^{32}P-c.p.m./10 ml. (**A**) *C.fasciculata*-minicircle conserved region probe (pDP312). (B–D) *C.helicis.* (**B**) Z4 minicircle probe. (**C**) cox 1 probe. (**D**) nuclear SSU rRNA probe.

C.fasciculata minicircle (pPK201/CAT) was used as a probe to screen the whole 4.2 kb Z4 insert. We subcloned and sequenced a 1.44 kb long fragment which contained sequence motifs characteristic of the trypanosomatid minicircles. In this sequence we identified two regions showing significant similarity to the invariant 12 bp universal minicircle sequence (CSB3) and an 8 bp conserved sequence (CSB2). Between CSB2 and CSB3, a region with high sequence similarity to the *C.fasciculata* minicircle bent helix was present. Also, two other bent helix-like regions that contained irregularly spaced A-tracts were identified (Figure 1C). Total *C.helicis* DNA digested with >30 restriction enzymes was hybridized separately with the Z4 probe and a probe prepared from the gel-isolated minicircle band. The hybridization patterns and the intensity of signal revealed high sequence homogeneity of the minicircle population and showed that the Z4 clone is a representative of a major minicircle class (data not shown).

The sedimentation properties of the *C.helicis* lysate centrifuged in a continuous sucrose gradient are presented in Figure 2. The obtained fractions were hybridized with the Z4 minicircle probe, a maxicircle probe [300 bp fragment of the cytochrome oxidase subunit 1 gene (cox1)] and a nuclear probe [2kb small subunit rRNA gene (SSU rRNA)]. The cell lysate of *C.fasciculata* hybridized with the pDP312 minicircle probe was used as a control (Figure 2A). Both the mitochondrial and nuclear probes proved the absence of a large network in the *C.helicis* genome (Figure 2B–D). Poor sedimentation, and thus retention, of most of the signal in the upper fractions corresponds with the expected individual status of the majority of mini- and maxicircles. It should be noted that minor peaks in the central part of the gradient of Figure 2B were indicative of possible small minicircle catenanes. A count retention experiment was performed by comparison of the hybridization signal between serially diluted total DNA and the sum of the signals of individual fractions. For both *C.helicis* and *C.fasciculata,* it ranged from 70–80%. These relatively low values are most likely due to a partial loss of DNA during precipitation of fractions prior to their loading onto membrane.

Together, the analysis of DNA by agarose gel electrophoresis (Figure 1A and B), the use of cell lysates in the propidium iodide gradient (data not shown), and the results from sucrose gradient (Figure 2) strongly indicate the absence of large networks in *C.helicis*. However, because the kDNA differs among species with respect to its fragility (Shapiro and Englund, 1995), and because a *C.helicis* network might be extremely sensitive to pipetting and shearing, the above methods might fail to prove its existence and only detect its breakdown products. To avoid any manipulation with the lysate or the nucleic acid, cells embedded in the low-melting agarose were subjected to pulse-field gel electrophoresis (PFGE). As shown in Figure 3B, lane 1, the large majority (~90% in different experiments) of the minicircles entered the gel, individual bands representing different single and catenated species. The small amount (<10%) of the minicircles that stayed in the slot most likely represent those minicircles that could not leave the block due to the interference of poorly lysed membranes and cell debris etc. Under the same conditions, a large majority of minicircles of stationary phase *C.fasciculata* cells stayed in the slot (Figure 3B, lane 3).

Electron microscopy of the pan-kDNA

In order to verify the existence of various forms of minicircles that we detected in agarose gels and hybridization studies, we resorted to electron microscopical analysis of *C.helicis* DNA. Only carefully isolated DNA that did not come into contact with ethidium bromide or other intercalating agents was studied. By the contour measure-

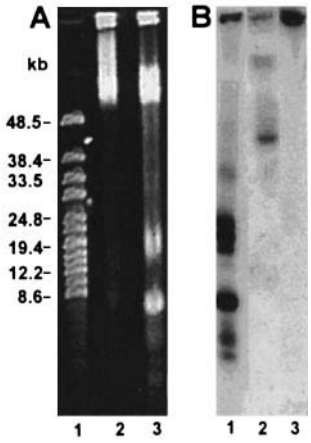

Fig. 3. PFGE of agarose-embedded cells was carried out as described in Materials and methods. (**A**) Ethidium bromide-stained agarose gel. CHEF size standard (lane 1); *T.borreli* (lane 2); *C.helicis* (lane 3); (**B**) Southern blot. *C.helicis* DNA was hybridized with Z4 minicircle probe (lane 1) and cox2 maxicircle probe (lane 2); *C.fasciculata* DNA was hybridized with pDP312 minicircle probe (lane 3).

ment of a representative number of circular molecules we confirmed that the minicircle population consisted of 4.2 kb molecules only (Figures 1 and 4A–L). To perform accurate measurements, most of the measured molecules were OC forms.

Among the 398 analyzed pan-kDNA components on the grids, we were able to find all the predicted minicircle forms, e.g. the SC (n=328) and OC monomers (n=19) (Figure 4A), the SC–SC (n=18), OC–SC (n=6) and OC–OC (n=7) dimers (Figure 4B–D), all the possible combinations of SC and OC molecules catenated into trimers (n=12) (Figure 4E–G), as well as rare larger catenanes (n=8). As shown in Figure 4I–L, the latter included catenanes up to the size of octamers. They were either composed solely of OC or SC forms, or both forms catenated at random (Figures 4I–L). In most cases, the neighbors were connected by a single interlock, as in the trypanosomatid kDNA networks. Two unusual structures that would not fit into the predicted *C.fasciculata* network model (Chen *et al.*, 1995a,b) were detected only once: a trimer with minicircles mutually interlocked into a triangle (Figure 4H) and a minicircle connected with four OC neighbours by single interlocks (Figure 4K). Although in these cases the localization of circles in different planes was not rigorously excluded, this possibility can be considered unlikely due to the low concentration of DNA, and especially the physical repulsion of DNA on grids (Fergusson and Davis, 1978). To exclude the possible disruption of larger catenanes during the preparation of DNA samples, the kDNA networks of *Trypanosoma carassii* were used as a control (Figure 4M).

In a group of 398 pan-kDNA components counted on randomly chosen sections of the grid, 82% and 5% were the SC and OC monomers, respectively; the SC–SC, SC–OC and OC–OC dimers together comprised 8%, and oligomers represented only about 5% of the analyzed molecules. In the agarose gels (Figure 1, lane 2), supercoiled minicircles also were the dominating species in untreated samples.

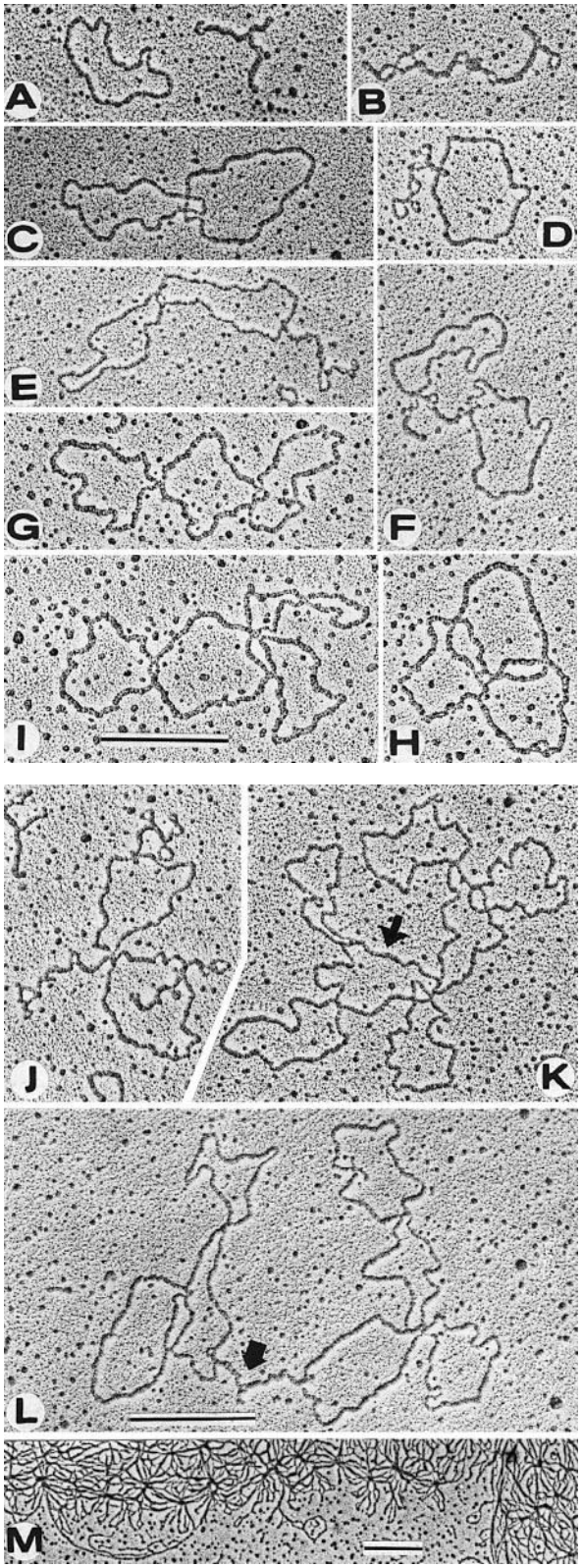

Fig. 4. Electron microscopy of the pan-kDNA. (**A**) OC and SC monomers. (**B**) SC dimer. (**C**) OC dimer. (**D**) OC–SC dimer. (**E**) OC–OC–SC trimer. (**F**) OC–SC–OC trimer. (**G**) OC trimer. (**H**) OC trimer with neighbor minicircles catenated as a triangle. (**I**) OC tetramer. (**J**) pentamer composed of three SC and two OC minicircles. (**K**) OC heptamer with a central minicircle (arrow) joint with four neighbors. (**L**) octamer composed of seven OC and one SC minicircles (arrow). (**M**) kDNA network of *T.carassii*. The magnification of all panels is equal except panel **M**; bars = 0.5 nm (~1.6 kb).

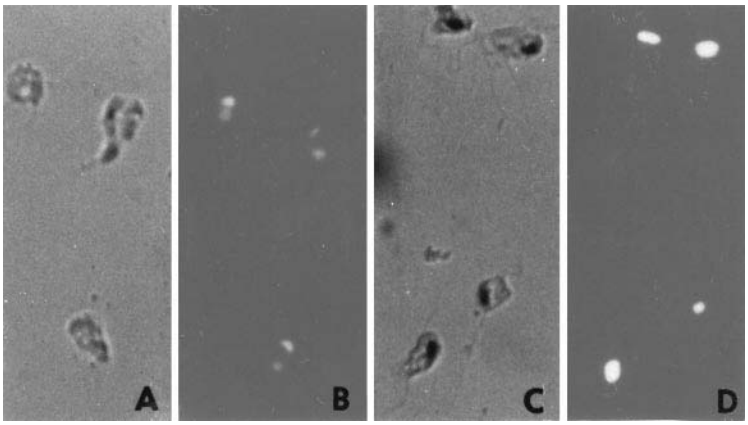

Fig. 5. Localization of the pan-kDNA minicircles in *C.helicis*. (**A**) Methanol-fixed and (**B**) propidium iodide-stained cells with round nucleus and rod-shape kinetoplast. (**C**) the pan-kDNA visualized by *in situ* hybridization with the Z4 minicircle probe. (**D**) The same cells as in (C) co-stained with propidium iodide that binds only to the nucleus.

Localization of the pan-kDNA in the mitochondrion

When trypanosomatid cells are stained for DNA, the kDNA appears as a small dot positioned adjacent to the flagellar kinetosome (Robinson and Gull, 1994). In contrast, when the *C.helicis* cell was stained with DAPI or propidium iodide, the pan-kDNA appeared as a prominent rod-shape structure, staining as intensely as the nucleus (Figure 5A and B). This is a strong indication that the pan-kDNA is distributed throughout the mitochondrial matrix. To exclude the possibility that bands considered as the pan-kDNA minicircles were in fact plasmids of cytoplasmic origin, we performed the *in situ* hybridization with the Z4 minicircle probe. As shown in Figure 5C, the probe hybridized with the mitochondrion only, thus confirming the organellar localization of the circles. As a control, the *in situ* hybridized cells were co-stained with propidium iodide (Figure 5D).

To investigate further the distribution of pan-kDNA in the mitochondrion, we studied the fine structure of the organelle in osmium-fixed and Epon-Araldite-embedded cells, this procedure enabling optimal preservation of the ultrastructure. In all the analyzed cells, a single elongated mitochondrion contained two prominent structures: short tubular-shaped cristae that stretched from the periphery into the matrix and multiple electron-lucent loci distributed evenly in the matrix (Figure 6A–D). These structures were not analyzed in detail, but the negative results of the polysaccharide-specific Thiery-staining indicated their protein composition (data not shown). In order to localize the pan-kDNA at the ultrastructural level, we fixed the cells with paraformaldehyde and embedded them in LR White resin. Mildly deproteinized ultrathin sections were treated with the α-DNA antibodies and secondary antibodies coupled with colloidal gold. As can be seen in Figure 6B–D, the colloidal gold particles were spread in the mitochondrial matrix. However, they were bound only to the electron-lucent loci, while no signal was associated with the tubular cristae or the periphery of the organelle.

Maxicircles and the abundance of pan-kDNA components

For the detection of maxicircles in the pan-kDNA, we PCR-amplified and cloned a fragment of the *C.helicis* cox2 gene and used it as a probe. To avoid hydrodynamic shearing of large maxicircles, we embedded cells in agarose blocks and used PFGE (see Materials and methods). Most of the maxicircle signal migrated at approximately 43 kb, indicating that, similar to the minicircles, there is no maxicircle network (Figure 3B, lane 2). The topology of maxicircles (circularity or linearity) has not been investigated. We also cloned and sequenced internal part of the cox1 gene which was, according to the hybridization studies (data not shown), situated adjacent to the cox2 gene.

Quantitative dot blot analysis was performed to determine the relative abundance of the maxi- and minicircles (Figure 7). As a reference, we used a plasmid construct, in which ~300 bp fragments of the nuclear LSU rRNA gene, the cox 1 gene and the conserved bent helix region of Z4 minicircle (Y6) were cloned in tandem. Because a single-copy gene of *C.helicis* is currently not available, we used as a copy number reference the nuclear LSU rRNA gene, which is known to be present in 100–200 copies in related flagellates (Leon *et al.*, 1978; Castro *et al.*, 1981; L.Floeter-Winter, personal communication).

Assuming that there were 150 copies of the LSU rRNA gene in the *C.helicis* genome, we calculated that there were 14 cox1 genes (and thus 14 maxicircles) and 8400 minicircles (considering that, as in trypanosomatids, each minicircle contains a single conserved bent helix region). The ratio between maxi- and minicircles hence was 1:600, and the total amount of mitochondrial DNA was ~35 Mbp.

In order to determine the relative amount of the pan-kDNA, non-dividing cells were stained by Feulgen reaction and the intensity of the signal was quantified by confocal microscopy as a sum of measurements in five arbitrarily chosen planes. By measuring the ratio between the mitochondrial and nuclear DNAs, we found that on average ~36% (33–41%) of the total cellular DNA was localized in the mitochondrion. Based on these values, the total genome size of *C.helicis* appeared to be about ~Mbp, this size being in good agreement with that obtained for related *T.borreli* (Yasuhira and Simpson, 1996).

Discussion

Cryptobia helicis was chosen as an organism useful for evolutionary studies because it fulfils the following criteria:

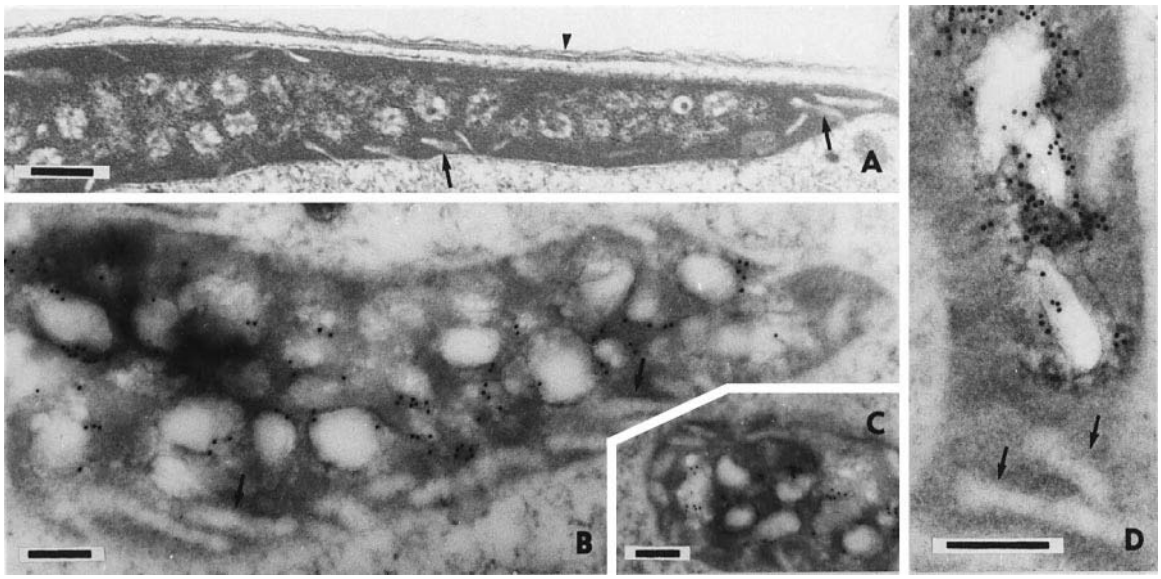

Fig. 6. Transmission electron microscopy and immunogold labelling. (**A**) The elongate mitochondrion in Epon-Araldite-embedded cells with peripheral cristae and multiple electron-lucent loci in its lumen. Longitudinal (**B** and **D**) and cross (**C**) sections of a mitochondrion in LR-White-embedded cells treated with the α-DNA antibodies and secondary antibodies coupled with colloidal gold. All the colloidal gold granules are associated with the electron-lucent loci while no DNA is detected at the peripheral cristae. Bars - 250 nm (A) and 100 nm (B–D) (arrows: mitochondrial cristae, arrowhead: cellular membrane).

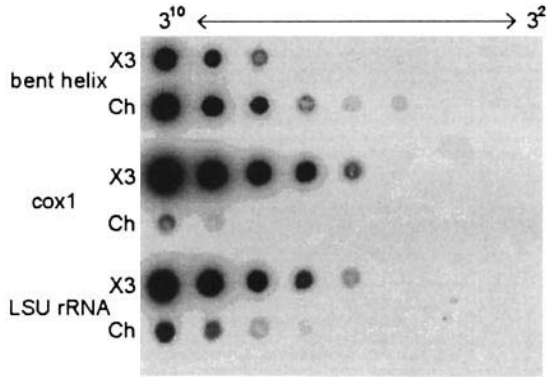

Fig. 7. Relative abundance of different *C.helicis* DNA components. Serial dilutions of a *Hha*I-digested genomic DNA (Ch) and an *Eco*RI-linearized reference plasmid (X3) (see Materials and methods) were hybridized with specific probes for each of the DNA components. The Y6 clone (the Z4 minicircle conserved bent helix); the cox1 gene (a maxicircle gene) and the LSU rRNA gene (a nuclear gene) are shown.

(i) it represents the earliest known evolutionary branch of the kinetoplastid lineage as deduced from the SSU and LSU rRNA sequences (D.Doležel, M.Jirků, D.A.Maslov, J.Lukeš, unpublished data); (ii) although with difficulties, it can be obtained pure in sufficient amounts, while all the other bodonids can only be cultivated with feeder bacteria or not at all (*T.borreli* being a single exception); and (iii) it contains the pan-kDNA.

Presence of the two prominent and several weak bands in the undigested samples of *C.helicis* DNA is indicative of either an extremely fragile network or free minicircles. However, the results obtained with the agarose-embedded cells run in a pulse field gel excluded the former possibility. In comparison, when DNA of the stationary phase *C.fasciculata* cells was analyzed under the same conditions, only ~1% of the minicircles entered the gel compared with ~90% of the *C.helicis* minicircles. Further support for the absence of a large network in *C.helicis* was provided by the centrifugation of various DNA components in sucrose gradient; in contrast to *C.fasciculata*, the minicircles did not reach the lower part of the gradient. On the contrary, the minicircle signal showed several minor peaks in the middle of the gradient, and increased to a major peak in the uppermost fraction. This distribution of minicircles in the gradient, when compared with the steep increase of the maxicircle and nuclear signals in the upper fractions, was indicative of small catenanes and free circles.

The kDNA minicircles of trypanosomatids represent the only nonsupercoiled circular DNA in nature (Rauch *et al.*, 1993). Unexpectedly, in *C.helicis*, most of the minicircle monomers and a significant part of dimers and oligomers are supercoiled. Supercoiled monomers represented the most abundant species followed by open circles. We do not know if these circles are covalently closed or they contain nicks or gaps, because the division of minicircles could not be studied due to apparent absence of the replication intermediates in the samples analyzed, which were prepared from the stationary phase *C.helicis* cells. We have also found catenanes composed of 2–8 minicircles, although catenanes larger than trimers were rare. With two exceptions, minicircles were always joined by a single interlock and members of the catenane were linked with up to four neighbours.

Replication of decatenated *Trypanosoma equiperdum* minicircles occurs via θ-intermediates, the daughter circles being segregated from one another by topoisomerase II (Ryan and Englund, 1989). Inhibition of this activity results in the appearance of oligomers, up to pentamers (Shapiro, 1994). We cannot exclude that the oligomers are products of an unusual minicircle replication or arise as a consequence of a local absence of topoisomerase II. However, only very speculative interpretations would explain the existence of some catenanes (Figures 4H–L) as replication intermediates.

Using the *C.fasciculata* minicircle probe, we subcloned

a 1.44 kb part of the *C.helicis* Z4 minicircle, in which regions with high homology to typical minicircle motifs were found. The putative CSB2 and CSB3 blocks differed by one and three mismatches, respectively, from the universally conserved CSB2 and CSB3 blocks of trypanosomatids (Ray, 1989; Simpson, 1997). A short but significant homology with the *C.fasciculata* bent helix was localized in the region between CSB2 and CSB3. In trypanosomatid minicircles, however, this characteristic sequence motif (Kitchin *et al.*, 1986) is invariably situated in the region outside the CSB2–CSB3 conserved region (Shapiro and Englund, 1995; Simpson, 1997). Furthermore, two other regions with irregularly spaced A-tracts were reminiscent of a bent helix sequence. The identified regions of similarity, although not very strong, indicate that the 4.2 kb circles already contain typical minicircle sequences and can therefore be considered to be equivalents to minicircles of modern flagellates.

We have also demonstrated that minicircles are distributed in the mitochondrial lumen, and not in a kDNA disc close to the flagellar kinetosome, as is the case in most kinetoplastids studied thus far. We were able to localize the DNA to the multiple electron-lucent structures in the elongate mitochondrion. The highest percentage of the mtDNA when compared to the total cellular DNA is known from trypanosomatids (15%) (Shapiro and Englund, 1995) and *Acanthamoeba castellanii* (up to 20%) (Burger *et al.*, 1995). In the mitochondrion of *C.helicis,* the pan-kDNA represents ~36% of the cell genome and thus might be the largest mitochondrial genome known.

The existence of a huge network of interlocked circles in the trypanosomatid mitochondrion puzzled investigators until the discovery of guide RNA (gRNA) genes coded by minicircles (Pollard *et al.*, 1990; Sturm and Simpson, 1990) and their function in editing of the maxicircle transcripts (reviewed by Simpson and Thiemann, 1995; Arts and Benne, 1996). Obviously, catenation and a sophisticated mechanism of the kDNA division might limit the potential loss of the minicircle-encoded gRNA genes during the mitochondrial division (Borst, 1991). *Trypanoplasma borreli*, in which minicircles are absent, solved the problem of losing the gRNA genes by placing them in tandem in a 180 kb circular molecule (Yasuhira and Simpson, 1996; Simpson, 1997).

The description of the pan-kDNA structure in *C.helicis*, which differs from the kDNA of morphologically closely related *T.borreli*, has a taxonomic consequence in final justification, after a three decade long taxonomic dispute (reviewed by Lom and Dyková, 1992), of the separate generic status of these flagellates.

In contrast to the kDNA minicircles, the pan-kDNA minicircles resemble plasmids by their monomeric status, supercoiling and loose distribution in the mitochondrial matrix. Based on this resemblance, an intriguing scenario can be drawn, in which minicircles might be descendants of a plasmid that invaded the mitochondrion of an ancient free-living bodonid. This mitochondrion was already equipped with its DNA, preserved until now in the form of a maxicircle. Multiple interactions of mitochondrial plasmids with each other and with mtDNA have been observed in the fungus *Neurospora crassa*, including the formation of transcript hybrids (reviewed by Griffith and Yang, 1995). After RNA editing arose by an as yet unknown mechanism (Simpson and Thiemann, 1995; Arts and Benne, 1996; Cavalier-Smith, 1997), mitochondrial plasmids possibly became involved, functioning as vehicles for the gRNA genes. A process of interlocking individual minicircles into a network proved to be an efficient way to prevent their loss during the mitochondrial division. RNA editing has not yet been demonstrated in the *C.helicis* mitochondrion. Although the cox2 gene and the internal part of the cox1 gene are not edited similar to the related *T.borreli* (Lukeš *et al.*, 1994; D.Blom, A.de Haan, M.Van den Berg, P.Sloof, M.Jirků, J.Lukeš, R.Benne, in preparation), and although homology search failed to find the gRNA genes in available minicircle sequences, the presence of RNA editing in this primitive flagellate can be expected. In that case, we can only speculate about the precise segregation mechanism of the dispersed minicircle population. Based on the estimation of various *C.helicis* DNA components mentioned above, the pan-kDNA contains about three times as much DNA as the kDNA of *C.fasciculata*, with minicircles accounting for this difference. Therefore, a redundancy of minicircles might be an alternative solution to the network structure. A challenge is to confirm, by a comparative analysis of the kDNA of primitive flagellates, that in *Cryptobia* such an ancient kDNA structure remained preserved.

Materials and methods

Organisms and DNA isolation

Cells of *C.helicis* were obtained from the pin-head sized receptacula seminis dissected from garden snails, *Helix pomatia*, captured in southern and central Bohemia, Czech Republic, and northern Italy. After opening the wall of the receptaculum, flagellates (about 2×10^7 cells/receptaculum) were carefully washed out by micromanipulation from the space between the receptaculum wall and the spermatophorous matrix. In most cases the flagellate population was in the stationary phase. *C.fasciculata* (Steinert strain), *T.carassii* (CC-Nem strain), and *T.borreli (*Tt-JH strain) were cultivated as described elsewhere (Kleisen *et al.*, 1975; Lukeš *et al.*, 1994; Jirků *et al.*, 1995).

Total DNA was isolated from cells after repeated washes in NET–50 (50 mM EDTA, 100 mM NaCl, 10 mM Tris, pH 8.0), lysed by N-lauroylsarcosine (Fluka) and pronase E (Merck) at the final concentrations of 3% (v/w) and 1 mg/ml, respectively, for 1 h at 4°C. The lysate was phenol-chloroform extracted and the DNA was ethanol-precipitated, air dried and resuspended in TE buffer. During the isolation, any vortexing and shearing was avoided.

Electrophoresis, blotting, hybridization and PCR

Agarose gel electrophoresis and blotting were performed according to standard protocols (Sambrook *et al.*, 1989). Low-melting agarose blocks with cells embedded at a concentration of either 4×10^7 (minicircle probe) or 4×10^8 (maxicircle probe) cells/ml were prepared as described elsewhere (Rovai *et al.*, 1992). PFGE was performed in a FIGE Mapper apparatus (Bio-Rad) in 1% agarose gel and 0.5× TBE buffer at forward and reverse voltages of 180V and 120V, respectively, on a linear switch time ramp (0.1–0.8 s) at 10°C for 11 h. Gels obtained from PFGE or regular agarose gel electrophoresis were blotted following treatment of the gel either with 0.5N HCl for 15 min or at 600 kJ at 254 nm, prior to denaturation and neutralization. With all the probes used, hybridization was performed at 65°C overnight, and the membranes were washed three times for 20 min in 3×SSC, 0.1% SDS at 65°C.

The cytochrome oxidase subunit (cox) 1 (410 bp) and cox 2 (330 bp) maxicircle probes were generated by PCR from genomic DNA using C112 and C115 oligonucleotides (Lukeš *et al.*, 1994) and oligonucleotides G566 (AAIGTIATIGGITTT[C]CAA[G]TG) and G567 (GGITTICAITG-A[G]TAT[C]TGA[G]GTITA), respectively. The fragments of small (2.0 kb) and large (1.0 kb) subunit ribosomal RNA (SSU and LSU rRNAs) nuclear genes were amplified using oligonucleotides S762 and S763, and S1842 and S1843 of Maslov *et al.* (1996), respectively. All PCR amplifications were performed as described elsewhere (Lukeš *et al.*,

1994; Maslov *et al.*, 1996). The conserved region minicircle probe pDP312 (Ferguson *et al.*, 1992) and the bent helix minicircle probe pPK201/CAT (Kitchin *et al.*, 1986) from *C.fasciculata* were kindly provided by L.Guilbride and P.T.Englund.

Restriction analysis and topology

Treatments with restriction endonucleases (Fermentas, Promega), DNase I (Boehringer Mannheim), topoisomerases I (Fermentas) and II (Amersham) were performed according to manufacturers' instructions. Treatment with Fe^{2+} ions reduced by hydrogen peroxide into OH-radicals, which degrade the deoxyribose and thus break the DNA backbone, was performed according to Fojta and Paleček (1997). Conditions of a representative experiment are described in Figure 1B.

Sucrose gradients

Approximately 6×10^7 cells of *C.helicis* (or 2×10^8 cells of *C.fasciculata*) were lysed as described above. The sample was diluted to 5 ml with water and loaded onto a 30 ml sucrose gradient prepared according to Englund (1979) and centrifuged at 7000 r.p.m. for 45 min at 4°C in a Sorvall HB–6 rotor. 1.4 ml fractions were collected from the bottom of the gradient using a Minipuls 2 gradient fractionator (Gilson). The fractions were diluted with 1.4 ml water, ethanol-precipitated, air-dried, redissolved in 100 µl water, spotted on Hybond-N (Amersham), and hybridized with the appropriate probes. The amount of signal was measured by phosphorimager Storm 860. Count retention was established by combination of the signal from all fractions and was compared with serially diluted total DNA hybridized with the same probe.

Cloning and sequencing of cox1 fragment and Z4 minicircle

A 410 bp PCR-amplified fragment of the cox1 gene was cloned into the *Eco*RV site of pT7blue vector (Novagen), and the *Bgl*II-linearized minicircle (Z4) was cloned into the *Bam*HI site of pBluescript SK(–) (Stratagene) and transformed in XL–1 competent cells (Stratagene). The cox1 fragment and the 1.44 kb long subclone of Z4 were sequenced using the Sequenase 2.0 kit (Amersham). The Z4 sequence has been deposited in GenBank under accession number AF034623.

In situ hybridization, confocal microscopy and DNA electron microscopy

For *in situ* hybridization, cells were attached to poly-L-lysine pretreated slides and fixed (4% formaldehyde; 5% acetic acid; 0.9% NaCl) for 5 min at room temperature. Z4 digested with *Hha*I was digoxigenin-labelled with the Dig-DNA labeling kit (Boehringer Mannheim) according to the manufacturers' instructions, and following the technique of Pearce *et al.* (1996). The detection of hybridized probes was performed with the Fab fragments of the sheep anti-digoxigenin antibody directly conjugated to the alcaline phosphatase (1:500 dilution in PBS) and its activity was detected with the BCIP/NBT substrate system. The cells were counterstained with propidium iodide (50 ng/ml) for 5 min at room temperature and examined in a Vario-Orthomat 2 (Leitz) microscope. Feulgen-stained smeared cells were examined in an Axiovert 100 (Carl Zeiss) confocal microscope. The ratio between nuclear and mitochondrial DNA was established as a sum of signals counted in five 0.2 nm thick arbitrarily chosen optical planes, separately for the nucleus and kinetoplast in 30 cells.

Total DNA of *C.helicis* was prepared for electron microscopy by the cytochrome-*c* method (Fergusson and Davis, 1978). Plasmid pBR322 and *T.carassii* kDNA served as standards. The samples were examined with a Philips CM12/STEM electron microscope at 80 kV. The contour lengths of the minicircles were measured from prints using the HIPAD digitizing tablet (Houston Instruments). The precise magnification was determined using replica granting (Balzers).

Immunogold labelling and transmission electron microscopy

Cryptobia helicis cells were fixed in 2% glutaradehyde in 0.2 M cacodylate buffer at 4°C overnight, postfixed in 2% osmium tetroxide in 0.2 M cacodylate buffer for 1 h at room temperature, embedded in Epon-Araldite and processed for electron microscopy as described elsewhere (Lukeš, 1992).

For immunogold labelling, cells were fixed in 2% paraformaldehyde in 0.2 M cacodylate buffer at 4°C overnight, rinsed three times in 0.2 M cacodylate buffer, pelleted, dehydrated, infiltrated and embedded in LR White resin at 60°C for 2 days. The blocks were thin-sectioned and the sections were picked up on 200 mesh copper grids with a carbon coated formvar membrane. The grids were placed in a moist chamber for the following incubations: 20 min in 2% proteinase K in phosphate buffered saline (PBS); 10 min in 0.5% fatty acid free bovine serum albumin; 1 h in α-DNA antibodies at room temperature; 20 min wash in PBS; 1 h in secondary antibodies (anti-mouse IgG) coupled with 10nm colloidal gold; 20 min wash in PBS; 30 min postfixation in 2% glutaraldehyde; five times 5 min wash in deionized water. The grids were poststained with 5% uranyl acetate and lead citrate, and examined in a JEOL JEM 1010 electron microscope.

Quantitation of DNA components

A 410 bp fragment of the cox1 gene, 290 bp of the Z4 minicircle fragment containing the conserved bent helix (Y6), and the 320 bp *Dra*I and *Hinc*II fragment of the LSU rRNA gene were cloned in tandem into appropriate restriction sites of the same plasmid, termed X3. In this plasmid, which was used as reference for quantitative dot blot analysis, the maxi- and minicircular and nuclear DNA fragments were present in equimolar amounts. *Hha*I-digested genomic DNA from *C.helicis* and *Eco*RI-linearized X3 DNA were serially diluted and spotted on Hybond-N membrane (Amersham). With each of the three DNA fragments that were labelled by nick translation, one of three identical blots was hybridized at 65°C overnight and washed three times 20 min in 3× SSC and 0.1% SDS. The experiment was performed four times.

Acknowledgements

The authors thank David Doležel, Mirek Fojta, Jiří Macaš, Jana Nebesářová, Marcela Nouzová, Artem Pliss, Radek Půr, and Ivo Šauman for discussions and help with various experiments, and Libor Grubhoffer for continuous support. The excellent technical assistance of Antonín Polák and Klárka Dudková is appreciated. We thank Paul T.Englund for his valuable comments and advice, and Rob Benne and Dmitri A.Maslov for critical reading of the manuscript. This work was supported by grants from the Grant Agency of the Czech Republic (204/94/0285) and the Grant Agency of the Czech Academy of Sciences (A6022503) to J.L.

References

Arts,G.J. and Benne,R. (1996) Mechanism and evolution of RNA editing in kinetoplastida. *Biochim. Biophys. Acta*, **1307**, 39–54.

Borst,P. (1991) Why kinetoplast DNA networks? *Trends Genet.*, **7**, 139–141.

Brugerolle,G., Lom,J., Nohýnková,E. and Joyon,L. (1979) Comparaison et évolution des structures cellulaires chez plusieurs espèces de Bodonidés et Cryptobiidés appartenant aux genres *Bodo*, *Cryptobia* et *Trypanoplasma* (Kinetoplastida, Mastigophora). *Protistologica*, **15**, 197–221.

Burger,G., Plante,I., Lonergan,K.M. and Gray,M.W. (1995) The mitochondrial DNA of the amoeboid protozoon, *Acanthamoeba castellanii*: complete sequence, gene content and genome organization. *J. Mol. Biol.*, **245**, 522–537.

Castro,C., Hernandez,R. and Castaneda,M. (1981) *Trypanosoma cruzi* ribosomal RNA: internal break in the large-molecular-mass species and number of genes. *Mol. Biochem. Parasitol.*, **2**, 219–233.

Cavalier-Smith,T. (1997) Cell and genome coevolution: facultative anaerobiosis, glycosomes and kinetoplastan RNA editing. *Trends Genet.*, **13**, 6–9.

Chen,J., Rauch,C.A., White,J.H., Englund,P.T. and Cozzarelli,N.R. (1995a) The topology of the kinetoplast DNA network. *Cell*, **80**, 61–69.

Chen,J., Englund,P.T. and Cozzarelli,N.R. (1995b) Changes in network topology during the replication of kinetoplast DNA. *EMBO J.*, **14**, 6339–6347.

Englund,P.T. (1979) Free minicircles of kinetoplast DNA in *Crithidia fasciculata*. *J. Biol. Chem.*, **254**, 4895–4900.

Ferguson,M., Torri,A.F., Ward,D.C. and Englund,P.T. (1992) In situ hybridization to the *Crithidia fasciculata* kinetoplast reveals two antipodal sites involved in kinetoplast DNA replication. *Cell*, **70**, 621–629.

Fergusson,L. and Davis,R.W. (1978) Quantitative electron microscopy of nucleic acids. In Koehler,J.K. (ed.), *Advanced Techniques in Biological Electron Microscopy II*. Springer Verlag, Berlin, pp.123–171.

Fernandes,A.P., Nelson,K., Beverley,S.M. (1993) Evolution of nuclear ribosomal RNA in kinetoplastid protozoa: perspectives on the age and origins of parasitism. *Proc. Natl Acad. Sci. USA,* **90**, 11608–11612.

Fojta,M. and Paleček,E. (1997) Supercoiled DNA modified mercury electrode: A highly sensitive tool for the detection of DNA damage. *Anal. Chim. Acta*, **342**, 1–12.

Gray,M.W. and Spencer,D.F. (1996) Organellar evolution. In

Roberts,D.M., Sharp,P., Alderson,P. and Collins,M. (eds), *Evolution of Microbial Life.* Cambridge University Press, Cambridge, pp.109–126.

Griffith,A.J.F. and Yang,X (1995) Recombination between heterologous linear and circularmitochondrial plasmids in the fungus *Neurospora. Mol. Gen. Genet*., **249**, 25–36.

Hajduk,S.L, Siqueira,A.M. and Vickerman,K. (1986) Kinetoplast DNA of *Bodo caudatus*: a noncatenated structure. *Mol. Cell. Biol*., **6**, 4372–4378.

Jirků,M., Kolesnikov,A.A., Benada,O. and Lukeš,J. (1995). Marine fish and ray trypanosomes have large kinetoplast minicircle DNA. *Mol. Biochem. Parasitol*., **73**, 279–283.

Kitchin,P.A., Klein,V.A., Ryan,K.A., Gann,K.L., Rauch,C.A. and Englund,P.T. (1986) A highly bent fragment of *Crithidia fasciculata* kinetoplast DNA. *J. Biol. Chem*., **261**, 11302–11309.

Kleisen,C.M., Borst,P. and Weijers,P.J. (1975) Properties of the intact multicircular complex from *Crithidia fasciculata. Biochem. Biophys. Acta*, **390**, 155–167

Leon,W., Fouts,D.L. and Manning,J. (1978) Sequence arrangement of the 16S and 26S rRNA genes in the pathogenic haemoflagellate *Leishmania donovani. Nucleic Acids Res.*, **5**, 491–504.

Lom,J. and Dyková,I. (1992) *Protozoan Parasites of Fishes.* Elsevier, Amsterdam, pp.48–64.

Lukeš,J. (1992) Life cycle of *Goussia pannonica* (Molnar, 1989) (Apicomplexa, Eimeriorina), an extracytoplasmic coccidium from the white bream, *Blicca bjoerkna*. *J. Protozool*., **39**, 484–494.

Lukeš,J., Arts,G.J., Van den Burg,J., De Haan,A., Opperdoes,F., Sloof,P. and Benne,R. (1994) Novel pattern of editing regions in mitochondrial transcripts of the cryptobiid *Trypanoplasma borreli. EMBO J*., **13**, 5086–5098.

Lukeš,J., Jirků,M., Doležel,D., Král′ová,I., Hollar,L. and Maslov,D.A. (1997) Analysis of ribosomal RNA genes suggests that trypanosomes are monophyletic. *J. Mol. Evol.*, **44**, 521–527.

Maslov,D.A., Avila,H.A., Lake,J.A. and Simpson,L. (1994) Evolution of RNA editing in kinetoplastid protozoa. *Nature*, **365**, 345–348.

Maslov,D.A. and Simpson,L. (1994) RNA editing and mitochondrial genomic organization in the cryptobiid kinetoplastid protozoan *Trypanoplasma borreli. Mol. Cell. Biol.*, **14**, 8174–8182.

Maslov,D.A., Lukeš,J., Jirků,M. and Simpson,L. (1996) Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa. *Mol. Biochem. Parasitol.*, **75**, 197–205.

Palmer,J.D. (1997) Organelle genomes: going, going, gone! *Science*, **275**, 790–791.

Pearce,S.R., Harrison,G., Li,D., Heslop-Harrison,J.S., Kumar,A. and Flavell,A.J. (1996). The T1-copia group retrotransposons in *Vicia* species: copy number, sequence heterogeneity and chromosomal localization. *Mol. Gen. Genet*., **250**, 305–315.

Pollard,V.W., Rohrer,S.P., Michelotti,E.F., Hancock,K. and Hajduk,S.L. (1990) Organization of minicircle genes for guide RNAs in *Trypanosoma brucei. Cell*, **63**, 783–790.

Rauch,C.A., Pérez-Morga,D., Cozzarelli,N.R. and Englund,P.T. (1993) The absence of supercoiling in kinetoplast DNA minicircles. *EMBO J*., **12**, 403–411.

Ray,D.S. (1989) Conserved sequence blocks in kinetoplast minicircles from diverse species of Trypanosomes. *Mol. Cell. Biol*., **9**, 1365–1367.

Robinson,D.R. and Gull,K. (1994) The configuration of DNA replication sites within the *Trypanosoma brucei* kinetoplast. *J. Cell Biol.*, **126**, 641–648.

Rovai,L., Tripp,C., Stuart,K. and Simpson,L. (1992) Recurrent polymorphisms in small chromosomes of *Leishmania tarentolae* after nutrient stress or subcloning. *Mol. Biochem. Parasitol*., **50**, 115–126.

Ryan,K.A. and Englund,P.T. (1989) Synthesis and processing of kinetoplast DNA minicircles in *Trypanosoma equiperdum. Mol. Cell. Biol*., **9**, 3212–3217.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor.

Shapiro,T.A. (1994) Mitochondrial topoisomerase II activity is essential for kinetoplast DNA minicircle segregation. *Mol. Cell. Biol.*, **14**, 3660–3667.

Shapiro,T.A. and Englund,P.T. (1995) The structure and replication of kinetoplast DNA. *Annu. Rev. Microbiol*., **49**, 117–143.

Simpson,L. (1997) The genomic organization of guide RNA genes in kinetoplastid protozoa: several conundrums and their solutions. *Mol. Biochem. Parasitol*., **86**, 133–141.

Simpson,L. and Thiemann,O.H. (1995) Sense from nonsense: RNA editing in mitochondria of kinetoplastid protozoa and slime molds. *Cell*, **81**, 837–840.

Sturm,N.R. and Simpson,L. (1990) Kinetoplast DNA minicircles encode guide RNAs for editing of cytochrome oxidase subunit III mRNA. *Cell*, **61**, 879–884.

Vickerman,K. (1976) The diversity of the kinetoplastid flagellates 1. In Lumsden,W.H.R. and Evans,D.A. (eds), *Biology of the Kinetoplastida*. Academic Press, London, pp.1–34.

Vickerman,K. (1977) DNA throughout the single mitochondrion of a kinetoplastid flagellate: observations on the ultrastructure of *Cryptobia vaginalis. J.Protozool*., **24**, 221–233.

Wiemer,E.A.C., Hannaert,V., Van den IJssel,P.R.L.A., Van Roy,J., Opperdoes,F.R. and Michels,P.A.M. (1995) Molecular analysis of glyceraldehyde–3-phosphate dehydrogenase in *Trypanoplasma borreli*: An evolutionary scenario of subcellular compartmentation in Kinetoplastida. *J. Mol. Evol*., **40**, 443–454.

Yasuhira,S. and Simpson,L. (1996) Guide RNAs and guide RNA genes in the cryptobiid kinetoplastid protozoan, *Trypanoplasma borreli. RNA*, **2**, 1153–1160.

Received September 4, 1997; revised and accepted November 27, 1997