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Research Brief

Cruzella marina (Bodonina, Kinetoplastida): non-catenated structure of poly-kinetoplast DNA☆

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Kinetoplast DNA (kDNA) is the most structurally complex mitochondrial DNA known. It is a hallmark of Kinetoplastida, a group of flagellates that belong to the primitive eukaryotes. kDNA usually consists of thousands of circular DNA molecules of two types—maxicircles and minicircles. Minicircles, present in several thousands copies per mitochondrion, are usually identical in size but heterogeneous in sequence. Maxicircles encode typical mitochondrial genes, some of which are present in an encrypted form. To generate functional proteins, the cryptic mRNAs undergo post-transcriptional modification via a uridine insertion/deletion-type RNA editing process, which is specified by the minicircle-encoded guide (g) RNAs (for review see Simpson et al., 2000; Stuart and Panigrahi, 2002).

The order Kinetoplastida is divided into the suborder Trypanosomatina with all species being obligatory parasitic, and the suborder Bodonina that embraces free-living, commensalic and parasitic flagellates. In the Trypanosomatina, minicircles and maxicircles are interlocked to form a single network of relaxed (not supercoiled) molecules. In vivo, the kDNA network is highly condensed in the periflagellar region of the mitochondrial matrix. This catenated format is conserved in all trypanosomatids studied so far (for review see Klingbeil et al., 2001). Interestingly, the early-branching bodonids (Doležel et al., 2000; Dyková et al., 2003; Simpson et al., 2002) contain an unexpected diversity in the topology and cellular organization of the kDNA. In fact, in their mitochondrion minicircles and maxicircles are present in a plethora of forms, none of which is a network. Recently, a comparative analysis of these forms resulted in a hypothesis that addresses evolution of the kDNA network (Lukeš et al., 2002). One of the primitive forms of the kDNA is the poly-kinetoplast DNA (poly-kDNA) format. Based on electron microscopy, poly-kDNA has been described in bodonids belonging to the genera *Ichthyobodo* (*Costia*), *Rhynchobodo*, *Hemistasia*, *Dimastigella* (Lukeš et al., 2002), and *Cruzella* (Frolov and Malysheva, 2002). However, on the DNA level its structure has only been studied in *Dimastigella trypaniformis* and *Dimastigella mimosa* (Štolba et al., 2001). Herein, we describe the structure of poly-kDNA in another bodonid—*Cruzella marina*, which belongs among the least known bodonids.

Cruzella marina isolated from the intestinal tract of the sea squirt captured in Pacific Ocean in 1991 was obtained from the American Type Culture Collection (strain ATCC 50326), and was cultivated with the feeder bacteria *Alcaligenes xylosoxidans* in the ATCC medium 1525. In order to prevent overgrowth by the feeder bacteria, cultivation was performed at 16 °C in volumes and depth not exceeding 50 ml and 0.5 cm, respectively. After the culture reached the cell density of about 2×10^6/ml, the medium was collected and centrifuged at 6000 rpm for 20 min. The white upper part of the pellet that contained bacteria was carefully removed, the residual lower part of the pellet was resuspended and subjected to several rounds of differential centrifugation in order to partially remove the feeder bacteria. Finally, total DNA was isolated from the lysed flagellates by phenol–chloroform extraction with vortexing being avoided.

Agarose electrophoresis of gels stained with ethidium bromide (EtBr) either before or after separation of the DNA was performed as described (Lukeš et al., 1998). When total undigested DNA was separated in 0.75% agarose gel containing 0.5 μg/ml EtBr, three bands of 4.4, 2.2, and 0.9 kb appeared in the lower part of the gel, along with the high molecular DNA that represents chromosomes of *C. marina* (Fig. 1A, lane 1). These bands may represent different size classes of the kDNA minicircles, various topological forms of a single minicircle class, or even bacterial plasmids. In order to exclude the latter possibility, we have isolated total DNA from the feeder bacteria after the separation of the *Cruzella* cells. This DNA does not contain any small circular molecules (Fig. 1C, lane 1), and its digestion by various restriction enzymes including *Cla*I resulted in a smear (Fig. 1C, lane 2).

Out of several hexanucleotide-target restriction enzymes, only *Cla*I linearized the putative minicircle (Fig. 1A, lane 2) which was subsequently cloned and sequenced in its entirety. The ~2 kb circular molecule (CM1 minicircle) is relatively AT poor (55%) and contains the phased homopolymeric dA tracts and the conserved sequence block II (positions 1975–1982), both motifs typical for the kinetoplastid minicircles (Klingbeil et al., 2001; Ray, 1989). However, the conserved sequence block III that serves as replication origin in trypanosomatids is absent. No open reading frame was identified in the CM1 minicircle, and similarities with gRNA genes of other kinetoplastids were insufficient for an assignment.

The CM1 minicircle was labeled by random priming and used as a probe in Southern hybridization. After filling the slots by agarose to ensure proper blotting of DNA trapped in the slot, the gel was exposed to 600 kJ at 254 nm to nick the circular DNA and blotted overnight at 4 °C. Hybridization was performed at 65 °C overnight, followed by

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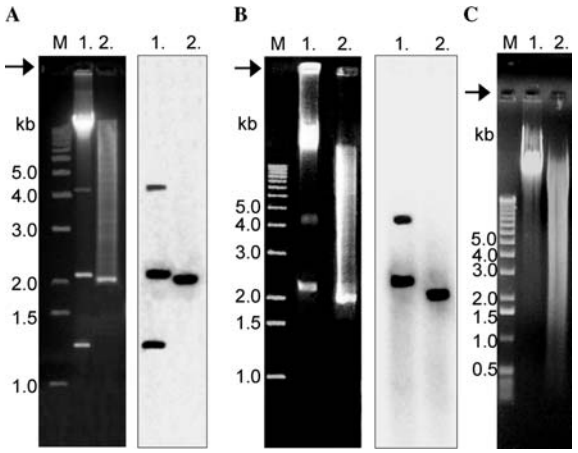

Fig. 1. Agarose gel electrophoresis of *Cruzella marina* (A, B) and the feeder bacteria *Alcaligenes xylosoxidans* (C). Undigested (lane 1) and *Cla*I-digested total *C. marina* DNA (lane 2) was run in the presence of EtBr (A), or in the absence of EtBr, and post-stained with 1 μg/ml EtBr for 1 h (B); undigested (lane 1) and *Cla*I-digested bacterial DNA (lane 2) was separated in the presence of EtBr (C); 1 kb ladder (lane M). The gels in (A) and (B) were blotted and hybridized with the CM1 minicircle probe.

three stringent washes in 3× SSC, 0.1% SDS at 65 °C. When undigested and digested DNA were hybridized with this clone, the signal co-migrated with three light bands, while no signal was present in the slot. Since *Cla*I linearized most of the minicircles, after the digestion the 4.4 and 0.9 kb bands disappeared, indicating that they represent minicircle dimers and supercoils, respectively. The linear and circular relaxed minicircles migrated as 2.0 and 2.2 kb bands, respectively (Fig. 1A).

To further test the topological status of the fast-migrating 0.9 kb band, total DNA was separated in the absence of EtBr and the gel was post-stained with the agent. Migration of the well-visible 2.2 kb band constituted from relaxed minicircles, and the 4.4. kb band composed of two mutually interlocked relaxed circles was not affected by the absence of EtBr. However, under these conditions the 0.9 kb band was absent (Fig. 1B). Its absence can be explained by artificial supercoiling induced during the run by EtBr to those minicircles that had both strands intact. Minicircles that contained nicks or gaps in one strand retained the relaxed topology even in the presence of the intercalation agent.

The total amount of 20 μg *C. marina* DNA was added to the CsCl-Hoechst 33258 equilibrium density gradient with the initial refractive index adjusted to 1.3950 that was centrifuged in a Beckman Ti50 rotor at 45,000 rpm for 72 h at 20 °C. Under these conditions, the DNA became separated into four distinct bands of different intensities (Fig. 2A). After dialysis, the DNA from individual bands was ethanol-precipitated, resuspended in distilled water, and hybridized under stringent conditions with the CM1 minicircle probe. As shown in Fig. 2B, the upper bands (fractions I and II) were highly enriched for kDNA.

Carefully isolated *C. marina* total DNA was spread by the cytochrome *c* method (Lukeš et al., 1998) and viewed in a JEOL JEM 1010 transmission electron microscope. Out of 279 circular ~2.0-kb long molecules examined, 94.9% were monomers, 4% dimers (Fig. 2C), 0.7% trimers, and 0.4% tetramers. We did find neither larger networks nor supercoiled molecules of different sizes. The kDNA network of *Crithidia fasciculata* prepared in parallel under the same conditions was intact (data not shown).

Both the results obtained by agarose gel electrophoresis and electron microscopy showed that in the pan-kDNA of *C. marina*, mini-

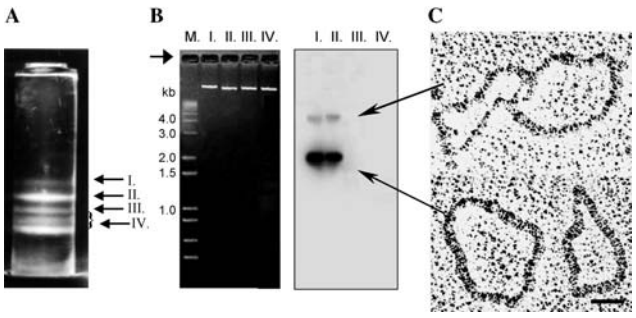

Fig. 2. Analysis of poly-kDNA from *C. marina*. CsCl-Hoechst 33258 equilibrium density gradient (A). Equal amounts of the DNA extracted from bands I to IV were loaded onto a 0.75% agarose gel, post-stained with EtBr after the run, blotted, and hybridized with the CM1 minicircle probe (B). Minicircle monomers (lower panel) and a dimer (upper panel) viewed in the transmission electron microscope (C). Bar indicates 50 nm.

circles are present as relaxed non-catenated molecules. In order to exclude the existence of a fragile kDNA network that would be disrupted during the isolation procedure, we have loaded lysed *Cruzella* cells on a sucrose gradient prepared as described elsewhere (Blom et al., 2000). After a short run that is sufficient for sedimentation of a large network to the bottom of the gradient, individual fractions of the gradient were screened with the CM1 minicircle probe. We interpret the presence of all the minicircle signals on top of the gradient (data not shown), as another line of evidence for the existence of free non-catenated minicircles in the kinetoplast of *C. marina*.

Cruzella marina is a poorly known parasitic/commensal flagellate, member of the monospecific genus *Cruzella*. Due to the lack of data, *Cruzella* appeared on the list of flagellates with unknown taxonomic affinities, being only tentatively ranked among bodonids (Patterson et al., 2002). However, a phylogenetic analysis based on the 18S rRNA gene sequence clearly proved its appurtenance to the order Kinetoplastida (Doležel et al., 2000; Simpson et al., 2002). Moreover, a recent ultrastructural analysis revealed typical bodonid features like two flagella and cytostome, and provided further support for classifying *Cruzella* into the suborder Bodonina (Frolov and Malysheva, 2002). Analysis of the available kinetoplastan 18S and 28S rRNA dataset with different methods and using different outgroups failed to resolve the branching order of early-diverging species, since it yielded alternative topologies with generally low bootstrap support. While in some analyses *Cruzella* constitutes one of the earliest branches within the order (Doležel et al., 2000), in others it appears as a derived organism (Callahan et al., 2002; Dyková et al., 2003). Still, its affiliation with the lineage consisting of *Bodo designis*, *B. saliens*, *Dimastigella* spp., *Rhynchobodo* sp., and *Rhynchomonas nasuta* is a common feature of all trees (Callahan et al., 2002; Doležel et al., 2000; Simpson et al., 2002). This diverse lineage embraces the least known bodonids, from which only *D. trypaniformis* and *D. mimosa* have so far been studied at the molecular level (Štolba et al., 2001).

Poly-kDNA distinguishes from the other kDNA structures by several features. Its minicircles are monomeric and covalently closed (CC) but not supercoiled. Moreover, they are condensed into multiple discrete loci throughout the mitochondrial lumen (Lukeš et al., 2002). According to the current scenario on the evolution of kDNA, poly-kDNA is a form derived in respect to the pan-kDNA and mega-kDNA of cryptobiids, while it seems to have predated the complex catenated format of trypanosomatids (Lukeš et al., 2002; Simpson et al., 2002). Our data show that all these features are present in the poly-kDNA of *C. marina* as well.

The CC topological form of circular DNA molecule has in nature been demonstrated only in the kDNA (Rauch et al., 1993). Interestingly, it is a feature shared by minicircles of kDNA, pro-kDNA, and

poly-kDNA, the latter of which is apparently the most ancestral form. By inducing artificial supercoiling to the poly-kDNA minicircles by EtBr, we were able to demonstrate that both strands of the DNA molecule are intact. Moreover, the comparative analysis of poly-kDNA of *Dimastigella* and *Cruzella* showed that the lack of catenation, open circular status of minicircles and their small size are features conserved for this arrangement of kDNA. It would be a challenge to check whether a key enzyme for the replication of kDNA–topoisomerase II, follows in poly-kDNA the distribution of non-catenated minicircles, as was recently shown in another kinetoplast composed of non-catenated relaxed circles (Gažiová and Lukeš, 2003).

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