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BERENIL INDUCES THE COMPLETE LOSS OF KINETOPLAST DNA SEQUENCES IN *Trypanosoma equiperdum*

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Summary. A fully kinetoplastic strain of *Trypanosoma equiperdum* has been made dyskinetoplastic by successive treatments of infected rats with berenil. The loss of kDNA sequences is stable and complete as proven by reassociation kinetics of *in vitro* labeled kDNA. Moreover these trypanosomes exhibit the same infectivity as the wild-type.

The kinetoplast DNA (kDNA) condensed in the single mitochondrion of the trypanosome can be seen in Giemsa or Feulgen stained preparations as a dense granule situated at the base of the flagellum. In *T. equiperdum* the kDNA is composed of about 3000 minicircles of 1 kilo base pairs (kb) and about 50 maxicircles of 23 kb linked together to form a large network of high molecular weight (1). This organization is common to all members of kinetoplastida studied so far (reviewed in 2,3). The biological function of kDNA is not yet understood. The maxicircles are transcribed and may contain genes similar to those of mitochondrial DNAs in other eukaryotic cells (4)(5). The minicircles are also transcribed. Recently an RNA molecule complementary to about 10 % of the minicircle was described in *Crithidia acanthocephali* (6).

Mutant trypanosomes with apparently defective kDNA have been often described (reviewed in 2,7,8). These mutants can occur spontaneously or be induced by drugs. In any case they lack the stainable kinetoplast granule and are called dyskinetoplastic mutants. The study of these mutant trypanosomes with defective kDNA may provide new information about its role.

In this work we have induced the loss of kDNA of *Trypanosoma equiperdum* in the bloodstream of infected rats by treatment with berenil, a potent trypanocidal drug (9). We present the results of reassociation kinetics of *in vitro* labeled kDNA mini and maxicircles from untreated trypanosomes in the presence of total cell DNA from berenil treated cells. We demonstrate the complete and irreversible loss of kDNA sequences in these trypanosomes (akDNA trypanosomes).

MATERIALS AND METHODS

Isolation of akDNA trypanosomes. Female Wistar rats of about 200 g were infected with a fully kinetoplastic strain of *Trypanosoma equiperdum* from the an-

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tigenic variant called BoTat 24 (Bordeaux-Trypanozoon-Antigenic Type 24). This variant was isolated and characterized as previously described (10). The number of trypanosomes injected to each rat was about 10^6 in order to obtain the maximum parasitemia in 3 to 4 days. Two days after injection of trypanosomes each rat received intraperitoneally 1 mg of berenil (4,4' diazo amino dibenzamidine diaceturate) in solution in 0.1 ml of 0.15 M NaCl. The rats were sacrificed 8 to 10 days later and their parasitized blood was used to infect new rats then treated with 1 mg of berenil in a similar manner. Successive infections and berenil treatments were continued 5 times. The trypanosomes obtained are considered as 100 % dyskinetoplastic from cytological staining , and are named ak-Teq ber. The trypanosomes were purified as described by Lanham and Godfrey (11) and washed three times with 0.15 M NaCl - 0.015 M Na_3 citrate.

Preparation of total DNA and fractionation of kDNA. Total DNA was extracted from the kinetoplastic and from akDNA strain as previously described (12). kDNA networks can either be purified in CsCl-Ethidium bromide (EthBr) gradient as covalently closed molecules or in the presence of the bisbenzimidazole dye Hoechst 33258 in conditions previously described (13). This dye enhances the separation on the basis of its preferential binding to AT rich DNA (14).

Analytical ultracentrifugation. A Spinco model E analytical ultracentrifugation was used. Equilibrium density centrifugation in CsCl gradient was performed during 24 h at 44 000 rpm and 25°C with *Micrococcus lysodeikticus* DNA as density marker. Scans were obtained at 265 nm using the Beckman photoelectric scanning system.

In vitro synthesis of ^{3}H-labeled kDNA and reassociation kinetics. After incubation of kDNA network with *Bam* H1 restriction endonuclease, the maxicircles are cleaved in one linear fragment of 23 kb and the minicircles which are not cleaved are maintained intact under a core-kDNA network form I (1). The minicircles and linearized maxicircles are fractionated in a CsCl-EthBr gradient and then purified in a CsCl-Hoescht 33258 dye gradient as described above. The minicircles were cleaved into linear fragments of 1 kb size by *Hinf* I endonuclease (15). Both components of kDNA were nick-translated as described by Mackey *et al.* (16) in the presence of [^{3}H] dCTP (Amersham, specific activity 19 Ci/mmole) and [^{3}H] TTP (Amersham, specific activity 41 Ci/mmole). The size of the nick-translated DNA fragments was estimated by comparison with sheared ^{3}H-labeled *Trypanosoma cruzi* nuclear DNA (nDNA) of known size ($S^\circ_{20,w}$ = 5.9). The DNA polymerase I was purchased from Boehringer-Mannheim, the DNase I from Worthington, and the restriction endonucleases from Biolabs. The labeled DNAs, which gave at alkaline pH single-stranded fragments of about 400 nucleotides, were treated as described by Pellicer *et al.* (17) to remove the fast reannealing material (about 20 % of the probe DNA). ^{3}H-labeled DNAs were heated for 20 min. at 100°C in 30 mM Na phosphate pH 6.8 and reassociation was carried out at 68°C in 0.86 M NaCl, 80 mM Na phosphate pH 6.8 and 0.08 % SDS. Aliquots of 100 µl fractions were collected at various times and diluted with 0.9 ml of 0.14 M Na phosphate pH 6.8, 0.4 % SDS. The fractions of single and double-stranded DNA was determined by hydroxyapatite chromatography as described by Sharp *et al.* (18). About 90 % of probe minicircle and 85 % of probe maxicircle were capable of reassociation at saturation.

RESULTS AND DISCUSSION

When the total DNA extracted from *T. equiperdum* BoTat 24, is analysed by equilibrium density centrifugation in CsCl gradient (fig. 1A), the kDNA with a density of ρ = 1.690 g/cm3 and nDNA banding at ρ = 1.707 g/cm3 are observed as previously described (1). The kDNA which amounts to about 6 % of total DNA can

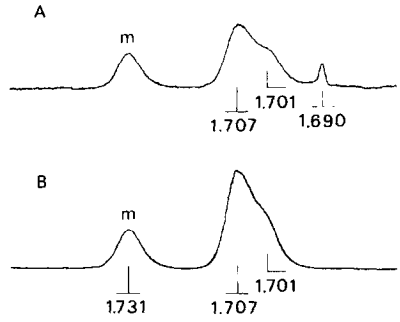

Figure 1. Equilibrium density gradient centrifugation in CsCl of *T. equiperdum* DNA. (A) Total DNA of BoTat 24. (B) Total DNA of berenil-treated trypanosomes (ak-Teq ber).

be isolated from total DNA in the form of covalently closed, high molecular weight network by preparative CsCl-EthBr gradient. The kDNA which is richer in AT than nDNA can also be efficiently fractionated in the upper band of a CsCl-Hoechst 33258 dye gradient (photograph not shown). When the total DNA extracted from ak-Teq ber trypanosomes was analysed by isopycnic CsCl gradient centrifugation, no trace of kDNA of density ρ = 1.690 g/cm3 was found (fig. 1B). Neither CsCl-EthBr nor CsCl-Hoechst dye gradients showed the usual kDNA band. Extensive examination of the DNA at the electron microscope does not reveal the presence of minicircles or maxicircles. Furthermore typical kDNA structures of *T. equiperdum* (1) could not be seen in ak-Teq ber trypanosomes even after extensive examination of ultrathin sections.

These data suggest that the kDNA usually found has been lost in ak-Teq ber trypanosomes. To confirm the complete loss of kDNA sequences in these dyskinetoplastic trypanosomes we have studied the reassociation kinetics of *in vitro* labeled kDNA mini and maxicircle from untreated trypanosomes with total DNA from berenil-treated trypanosomes. The molecular weight of *T. equiperdum* nDNA is about 4.3×10^{10} (19). The molecular weight of the kDNA minicircle is about 6.4×10^{5} (1). We have performed the reassociation kinetics under such conditions that the equivalent of one kDNA minicircle (in probe DNA) is incubated in the hybridization mixture with the equivalent of 2.6 total cellular genome of ak-Teq ber cells. The reannealing of labeled kDNA minicircles alone (control) and in the presence of 1 fold molar excess of unlabeled kDNA minicircles follows a simple second order kinetics as previously shown (1). The results presented in figure 2A show that the kinetics of reassociation of the labeled kDNA minicircle are not significantly modified by the unlabeled DNA from ak-Teq ber trypanosomes. Similar experiments have been carried out using *in vitro* ^{3}H labeled kDNA maxicircle fragments. The reannealing reaction follows also a simple second order kinetics as previously shown (1). The kinetics of reassociation of maxicircles,molecular weight 15.4×10^{6} (1) has been studied in the presence of the

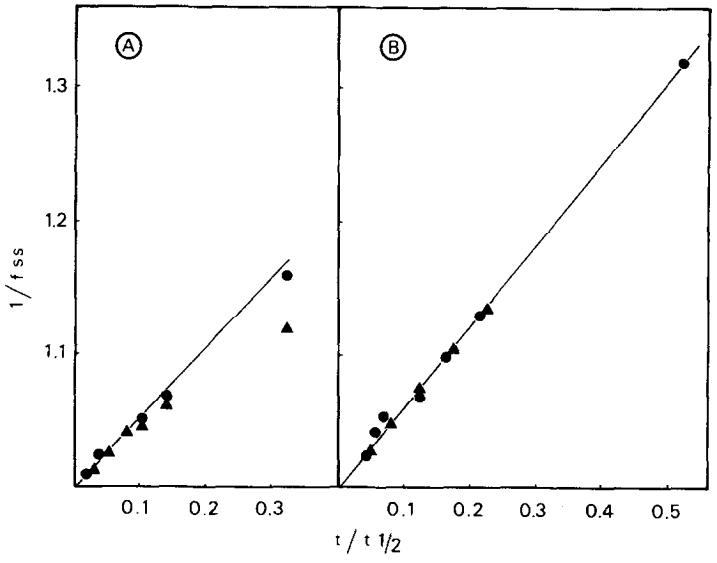

Figure 2. Reassociation kinetics of *in vitro* ^{3}H-labeled kDNA minicircle (A) and maxicircle (B) fragments from *T. equiperdum* BoTat 24 in the presence of total DNA from berenil-treated trypanosomes. (A) The reaction mixtures contained 2 ng/ml of labeled minicircle DNA (specific activity 7.4 x 10^6 cpm/µg) : alone (—●—) or in the presence of 344 µg/ml of unlabeled DNA from berenil-treated trypanosomes (—▲—). The time required for the reannealing of half of the ^{3}H-labeled DNA alone was 72 h. (B) The reaction mixtures contained 5 ng/ml of labeled maxicircle DNA (specific activity 1.5 x 10^7 cpm/µg), alone (—●—), or in the presence of 177 µg/ml of unlabeled DNA from berenil-treated trypanosomes (—▲—). The time required for the reannealing of half of the ^{3}H-labeled DNA alone was 46 h. The unlabeled fragments were prepared by sonication and contained 400 to 500 nucleotides, as measured by analytical ultracentrifugation at alkaline pH. The data are plotted according to Sharp *et al.* (18) where $1/f_{ss}$ represents the reciprocal of the fraction of probe DNA which is single-stranded at time t, and t ½ is the time required for half of the probe DNA to renature alone or in the presence of berenil-treated cells DNA.

equivalent of 12.7 total cellular genome from ak-Teq ber per equivalent of one kDNA maxicircle (in probe DNA). The results are not significantly different from the control (figure 2B). From these experiments we conclude that ak-Teq ber trypanosomes are akinetoplastic DNA (akDNA) cells.

Berenil which is a potent trypanocidal drug (9) has a specificity for base composition and does not intercalate between base pairs (20)(21). This drug induces dyskinetoplasty in trypanosomes and inhibits kDNA replication (9)(22). In kDNA isolated from *T. cruzi* treated with berenil many three-branched minicircles of the Cairns-type have been detected and assumed as being replicative intermediates (22).

In this paper we have shown after equilibrium density CsCl gradients that berenil induces the loss of kDNA structures in *T. equiperdum*. Furthermore, the reassociation kinetics of *in vitro*-labeled kDNA mini and maxicircles indicates the absence of kDNA sequences in the total DNA of berenil-treated cells. The

complete loss of kDNA is stable for at least 6 additionnal passages in rats, in the absence of berenil (maximum tested). The infectivity of these trypanosomes has been studied in mice and rats and no difference has been found with the kinetoplastic strain. The results we presented in this paper show that both components of kDNA complex networks are not apparently essential to the viability of bloodstream *T. equiperdum* . Similar results were obtained with *T. equiperdum* treated by EthBr and acriflavine (19). The problem of the biological function of kDNA remains open.

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