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RESEARCH NOTE

EVIDENCE OF ABSENCE OF *TRYPANOSOMA CRUZI* KINETOPLAST DNA METHYLATION BY RESTRICTION ENDONUCLEASE ANALYSIS

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Abstract—MUÑOZ S., VENEGAS J. and SOLARI A. 1991. Evidence of absence of *Trypanosoma cruzi* kinetoplast DNA methylation by restriction endonuclease analysis. *International Journal for Parasitology* **21**: 863–866. Eight kinetoplast DNA samples from very different *T. cruzi* zymodemes were digested with the isoschizomer group of enzymes (*Msp*I–*Hpa*II) and (*Mbo*I–*Sau* 3AI), able to detect DNA methylation on cytidine and adenine for the CCGG and GATC sequences, respectively. Restriction digestion analysis of each kDNA with both isoschizomer groups of enzymes did not display a different profile suggesting that maxicircles and minicircles on this trypanosomatid are not methylated.

INDEX KEY WORDS: Kinetoplast DNA; 5-methyl cytidine; 6-methyl adenine; *T. cruzi*; isoschizomers.

THE parasite *Trypanosoma cruzi* is the etiological agent of Chagas' disease in Latin America, directly affecting some 20 million people with perhaps 200 million more people having a high risk of infection (Brener, 1980). DNA methylation is a common feature in nature. In eukaryotes there is a striking inverse correlation between the levels of methylation of specific DNA segments and the extent to which these segments are expressed. Methylation of DNA on cytosine residues (CG sequence) can often influence nuclear gene expression in vertebrate cells where the extent of modification can be a high fraction of total cytosine content (van der Ploeg & Flavell, 1980). Methylation of DNA has also been shown to exist in lower eukaryotes such as protozoa. However, the subcellular localization of this nuclear or mitochondrial DNA modification is not always easy to assign. In *T. brucei* the evidence suggests that cytosine modification occurs in the telomeric area where the variable-specific antigens are expressed (Reibaud, Gaillard, Longacre, Buck, Bernardi & Eisen, 1983). Other indirect evidence for DNA modification in trypanosomatids comes from the observation of polymorphism in restriction cleavage by endonucleases having a GC sequence-specificity (Pays, Delauwe, Laurent & Steinert, 1984). However, nucleosides are absent in the nuclear DNA in *T. cruzi* after either enzymatic digestion (Crotazier, jan de Brij, den Engelse, Johnson & Borst, 1988) or restriction endonuclease analysis (Borst, Fase-Fowler, Frasch, Holijmakers & Weijers, 1980). Other authors working on *T. cruzi* found 5-methyl cytidine (5mC) and N6-methyl adenine (6mA) on epimastigote nuclear DNA after chemical hydrolysis (Rojas & Galanti, 1990). Trypanosomatid mitochondrial DNA has not been studied with respect to the covalent modification of DNA. These organisms, belonging to the order Kinetoplastidia, have an unusual mitochondrial DNA, the kinetoplast DNA (kDNA). The kDNA is formed by catenated circles that represent about 20% of the total cell DNA. Two types of circular DNA compose the kDNA network: the maxicircle, which is 16 kb pairs long, coding for some mitochondrial functions is present in 50 identical copies per cell, and the minicircle which is 1.45 kb long, heterogeneous in sequence, is present in 3×10^3–3×10^4 copies and whose function is beginning to be elucidated (Pollard, Rohner, Michelotti, Hancock & Hajduk, 1990). Some understanding of the way maxicircles may code for mRNA and the functioning of 'editosomes' is being achieved by minicircle transcripts (Sturm & Simpson, 1990). In this work we used restriction endonuclease isoschizomers to measure the extent of methylation of cytosine and adenine residues of the kDNA of eight *T. cruzi* populations with very different genetic backgrounds. A discussion about the possible regulation of kinetoplast expression genes is presented.

T. cruzi parasites were isolated from Chile and Perú by direct culture of infected bug feces in biphasic blood agar culture medium (Miles, Apt, Widmer & Schofield, 1984). All *T. cruzi* isolates were subsequently grown in bulk in a liquid medium as previously described (Carreño, Rojas, Aguilera, Apt, Miles & Solari, 1987). kDNA was purified using published procedures (Goncalves, Nehme & Morel, 1984). However, some kDNA samples were obtained by a modified procedure which involved mild shearing of *T. cruzi* DNA by passage through a needle in

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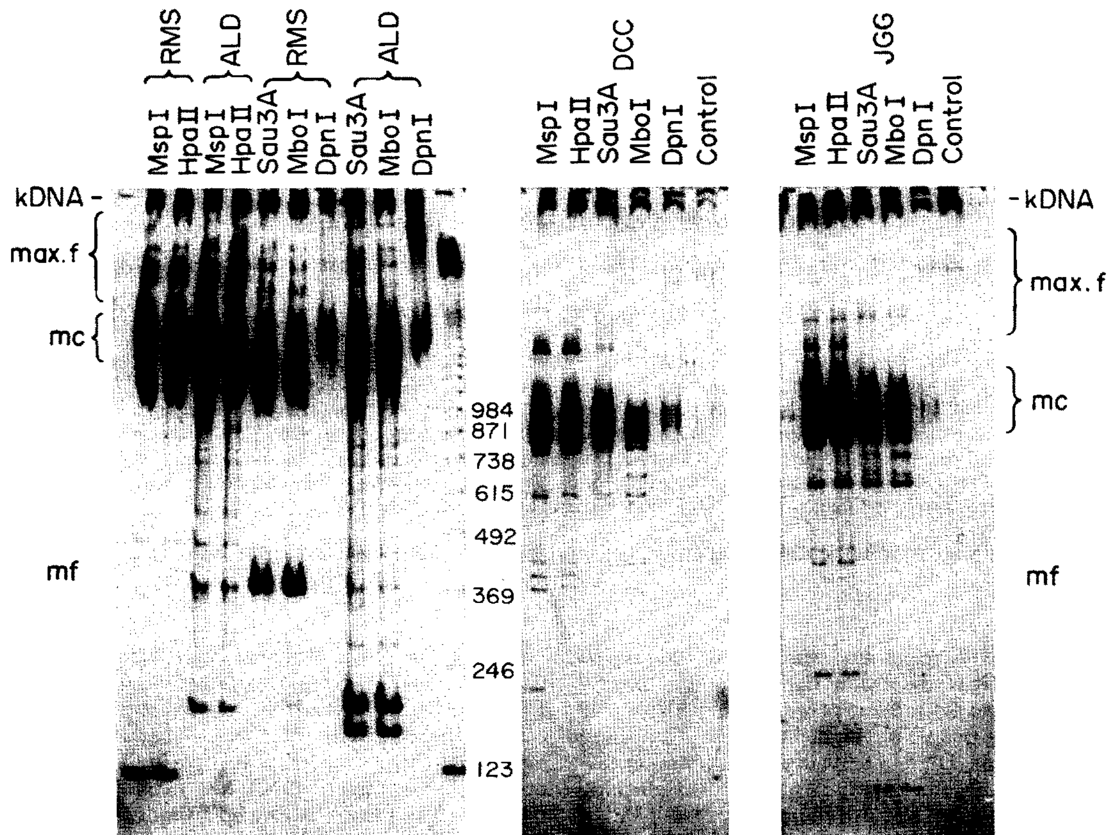

FIG. 1. Restriction endonuclease analysis of kDNA samples from *T. cruzi* isolates as described in the text. RMS, ALD, DCC and JGG are the *T. cruzi* kDNA samples. Each sample was cleaved with the restriction endonuclease indicated below. Left and right margins indicate different restriction endonuclease kDNA products. kDNA, endonuclease resistant kinetoplast DNA core; max. f, maxicircle fragments; mc, full-length minicircles; mf, minicircle fragments. The central line contains a molecular weight marker of 123 oligonucleotides and multiplicative oligonucleotides. Controls represent incubation of kDNA in the absence of enzyme. Polyacrylamide gradient gel electrophoresis and staining were performed as described in the text.

order to degrade nuclear DNA, followed by centrifugation to get the purified kDNA as a pellet. For endonuclease cleavage and gel electrophoresis 1 μg of kDNA was cleaved to completion with 1 unit of endonuclease under conditions specified by the manufacturer. Restriction fragments were separated by electrophoresis in 4.5–10% linear gradient polyacrylamide gels and further silver nitrate staining with a modified procedure (Goncalves *et al.*, 1984). Gels were preserved by vacuum drying between two sheets of cellophane paper. *Msp*I and *Hpa*II were obtained from Boehringer Mannheim. *Dpn*I, *Sau*3AI and *Mbo*I were obtained from BioLabs. Molecular weight standards used were a 123 bp DNA ladder and a ¢ × 174 DNA replicative form digested with *Hae*III. Eight *T. cruzi* populations were selected for a kDNA methylation study based on their restriction fragment length polymorphism with *Eco*RI and *Msp*I. Four of them correspond to the isoenzymatic profile named Zymodeme 2bol (RMS, ALD, JGG and DCC), three are Zymodeme 1 (v10P, LGN and Sp104) and one is Zymodeme 2bra (CEE). Two restriction endonuclease groups were chosen to cleave the kDNA samples. The first group was comprised of *Hpa*II and *Msp*I, both of which recognize the same sequence CCGG, except that *Hpa*II also recognizes the methylated sequence C5mCGG. The second group of isoschizomers, *Mbo*I and *Sau*3AI, recognizes the same sequence GATC, except that *Sau*3AI also recognizes the sequence G6mATC. The third enzyme in this group was *Dpn*I which cleaves the GATC sequence only if it is methylated, enabling a complete confirmation of adenine methylation on this particular sequence. A comparison of the restriction fragment pattern produced by cleavage of kDNA from Zymodeme 2bol parasites with *Hpa*II and *Msp*I is shown in Fig. 1. As expected several DNA bands were detected in the polyacrylamide gels. Each kDNA digested with *Hpa*II or *Msp*I gave similar patterns with maxicircle

and minicircle DNAs. This figure also shows that several kDNA samples do not generate maxicircle fragments upon enzymatic cleavage. The phenomenon occurs because maxicircle DNA is much more labile to shearing during kDNA purification. Each kDNA sample displays a typical restriction profile which defines the *T. cruzi* population. These profiles are called Schizodemes (Goncalves *et al.*, 1984). Restriction profiles from Zymodeme 1 and 2bra parasites also displayed similar patterns of DNA bands with each isoschizomer (not shown). Indistinguishable results with isoschizomers *Sau*3AI and *Mbo*I and kDNA from *T. cruzi* parasites were obtained as shown in Fig. 1. Moreover, *Dpn*I which recognizes that GATC sequence only if it is methylated in 6mA, did not cleave any kDNA samples. DNA fragments generated by these isoschizomers are mainly full-length minicircles, indicating that, on average, minicircles have only one GATC sequence. Maxicircle fragments on the other hand are also similar after digestion with both isoschizomers, although at a lower yield as compared with minicircles. The fact that some full-length minicircles are released upon enzymatic digestion is probably due to minicircle mechanical release and/or detachment from the kDNA network, since similar results are obtained after incubation without enzyme.

Maxicircle DNA of trypanosomatids encodes for mitochondrial functions. Some of these mitochondrial genes are transcribed and post-transcriptionally edited in a non-classical way (Feagin, Abraham & Stuart, 1988). Moreover, very recently it was found that minicircle transcripts exist and probably serve as guide RNAs for editing of mitochondrial transcripts (Pollard *et al.*, 1990). However, no information exists as to whether 5mC occurs and if this modification modulates maxicircle and minicircle gene expression as has been shown in the case of vertebrate nuclear genes. Our results indicate that a large number of minicircle fragments and a small number of maxicircle fragments were generated by both isoschizomer sets of enzyme. This way it is possible to approach the question as to whether a DNA is methylated or not based on the restriction endonucleases' specificity. We have previously shown that endonuclease digestion of kDNA with *Hpa*II and *Msp*I gave similar minicircle fragment DNA patterns from a variety of *T. cruzi* isolates (Carreño *et al.*, 1987). In the current paper we extend this type of analysis to other *T. cruzi* kDNA samples, including a second isoschizomer group of enzymes (*Mbo*I, *Sau*3AI and *Dpn*I). All this information suggests that maxicircles and minicircles are not methylated. However, this method of analysis indirectly measures DNA methylation and has an inherent limitation. Since only CCGG and GATC sites were studied, the samples of 5mC and 6mA sites were probably few and restricted to a small fraction of the total minicircles in the kDNA network. Several minicircles from *T. cruzi* have been sequenced from different clones and isolates of *T. cruzi* (Macina, Sanchez, Gluschankof, Burrone & Frasch, 1986; Degrave, Fragoso, Britto, van Heuverswyn, Kidane, Cardoso, Mueller, Simpson & Morel, 1988). We looked at these sequences and found that the *Hpa*II recognition sequence represents about 3.8–7.1% of the total CG sequences (with one and two restriction sites, respectively). This sequence is known as the exclusive site for cytosine methylation in the DNA of higher eukaryotes. This means that, if kDNA really is methylated in trypanosomatids the same way as in other eukaryotic nuclear systems, we are looking at only a small fraction of the methylatable sequences. However, this kind of analysis has been used successfully to study DNA methylation in other systems (van der Ploeg & Flavell, 1980).

All this information on restriction fragment length polymorphisms of kinetoplast DNA with two isoschizomer enzyme sets suggests that both maxicircle and minicircle DNA are not methylated in *T. cruzi.* Our failure to detect DNA methylation on *T. cruzi* kinetoplast DNA would mean that nucleoside modification does not play a role in the minicircle and maxicircle gene regulation expression. Also, no cytosine methylation has been detected in *T. brucei* nuclear DNA using the same pair of isoschizomers (Borst *et al.*, 1980). However, these observations do not preclude the possibility that kinetoplast and nuclear genes can be activated by a subtle control of DNA modification that may be below the detection limit of our analytical method.

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