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DNA methylation in *Trypanosoma cruzi*

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DNA isolated from the protozoan *Trypanosoma cruzi* has been found to contain 5-methylcytosine. Analysis of *T. cruzi* DNA by both *Hpa*II and *Msp*I restriction endonucleases suggests that the sequence -CCGG- is not methylated. Probably *T. cruzi* DNA also contains N^6-methyladenine. This report constitutes the first clear demonstration of the presence of methylated bases in the nuclear DNA from trypanosomes.

DNA methylation; 5-Methylcytosine; Base composition; *Trypanosoma cruzi*

1. INTRODUCTION

Trypanosoma cruzi, the agent of American trypanosomiasis or Chagas' disease, presents 3 phenotypes during its life cycle: amastigote (intracellular and replicative form in the host), epimastigote (extracellular and replicative form in the vector) and trypomastigote (extracellular, nonreplicative and infective form) [1]. Morphological, replicative and infective changes should be the result of modification in gene expression. In this regard, we have studied DNA methylation as a possible mechanism of regulation of gene activity in this protozoan.

Methylated bases should affect DNA-protein interactions as well as DNA conformation and thus could alter gene expression. There is a close correlation between genes which are actively expressed and their undermethylated condition [2,3].

It has been described that DNA from most organisms contains modified bases, usually 5-methylcytosine (m^{5}Cyt) or N^6-methyladenine (m^{6}Ade). Nuclear DNA from vertebrates presents only m^{5}Cyt preferentially in the sequence CpG, while that from unicellular eukaryotes contains m^{5}Cyt or m^{6}Ade or both minor bases [2–5].

Several reports have previously described the absence of methylated bases in DNA from African trypanosomes [6–9]. Here we present evidence supporting the conclusion that m^{5}Cyt, and probably m^{6}Ade, are present in *T. cruzi* DNA. Furthermore, we propose that m^{5}Cyt is in a restriction site different from CCGG.

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Abbreviations: m^{5}Cyt, 5-methylcytosine; m^{6}Ade, N^6-methyladenine; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; EDTA, ethylenediamine-tetraacetic acid; $_N$DNA, nuclear DNA; $_K$DNA, kinetoplast DNA; SDS, sodium dodecyl sulphate; 5-aza-C, 5-azacytidine

2. MATERIALS AND METHODS

2.1. *Cell culture*

T. cruzi strain Tulahuen was grown at 28°C in Diamond medium [10] supplemented with 2.5% fetal bovine serum. The cells were collected by centrifugation and washed in PBS, pH 7.2. *T. cruzi* strain RA was obtained from Dr A.C.C. Frasch, Fundación Campomar, Buenos Aires, Argentina.

2.2. *DNA labelling*

A procedure similar to the one described by Pratt and Hattman [4] was used. *T. cruzi* epimastigotes were continuously labelled during exponential growth (days 0–9) in the presence of 20 μCi/ml of [^{3}H-methyl)methionine (80 Ci/mmol) from Amersham). At day 9, an additional 10 μCi/ml of labelled methionine were added and the cells were incubated further to day 12, when they were collected and mixed with unlabelled cells that were used as a carrier for DNA extraction.

2.3. *5-Azacytidine treatment*

5-aza-C is an analog of cytosine which induces DNA demethylation [11]. *T. cruzi* epimastigotes were treated daily, for 14 days, with 10^{-6} M of this analog freshly prepared in distilled water each day.

2.4. *DNA preparation*

$_N$DNA was isolated by the procedure of Fairlamb et al. [12] as modified by Borst and Fase-Fowler [13], using the supernatant after sedimentation of $_K$DNA [14]. $_N$DNA was treated with ribonuclease and it was separated from ribonucleotides and ribonucleosides by gel filtration on a Sephadex G-50 column [15].

2.5. *Hydrolysis of DNA and base analysis*

Labelled or unlabelled DNA was hydrolyzed by the procedure described by Wyatt [16] and the free bases were analyzed by two-dimensional paper chromatography following the modified method of Pratt and Hattman [4]. Standard bases were used as markers for their location under ultraviolet light. The spots were cut out, the bases were eluted into vials [17] and the radioactivity counted in a Beckman LS 100C scintillation counter.

Nonradioactive hydrolyzed DNA was resuspended in 20 mM ammonium formate and analyzed by HPLC using a modification of the method of Farrance and Ivarie [18]. A Partisil 10 SCX Whatman column (250 × 4.6 mm) was used at 1 ml/min applying a 20 min linear

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Table I

Analysis of bases in *T. cruzi* DNA by paper chromatography

Base	cpm	R_F	
		1st solvent	2nd solvent
Guanine	0	0.10	0.27
Cytosine/methylcytosine	2567	0.20/0.26	0.53/0.59
Adenine	89	0.33	0.38
Methyladenine	0	0.60	0.53
Thymine	567	0.52	0.77

Aliquots of 1.5×10^6 cells were incubated for 9 days with 20 μCi/ml of [^{3}H-methyl]methionine. At day 9, additional 10 μCi/ml of the radioactive amino acid were supplied. At day 12, DNA was extracted and hydrolyzed in formic acid. Bases were separated by bidimensional chromatography. Each base was detected by ultraviolet light, eluted and the radioactivity measured by liquid scintillation counting

gradient of 12% methanol/20 mM ammonium formiate to 12% methanol/40 mM ammonium formiate, pH 4.0. Ultraviolet absorption was measured at 260 nm.

2.6. *Endonuclease digestion assays*

DNA was digested with restriction enzymes *Hpa*II (5 units/μg DNA) or *Msp*I (10 units/μg DNA) (obtained from BRL) for 4 h at 37°C. Both enzymes recognize the same sequence, CCGG. However, the methylated sequence CmCGG is resistant to *Hpa*II cleavage, while remaining susceptible to *Msp*I digestion. Lambda phage DNA was added to an aliquot of the incubation mixture as a control to test the completion of $_N$DNA digestion.

2.7. *Southern blot analysis*

DNA fragments were size-fractionated by electrophoresis through a 0.8% agarose gel and transferred overnight to nitrocellulose filters by the technique of Southern [19]. The filters were hybridized with ^{32}P-labelled nick-translated probes and autoradiographed at −70°C. The DNA probes used were total $_N$DNA from strain Tulahuen and clones 2, 7, 13 and 30 [20]. These clones contain an internal repeat element of variable length among the different clones.

3. RESULTS

Table I shows the distribution of radioactivity in the bases of *T. cruzi* DNA from epimastigotes incubated in the presence of [^{3}H-methyl]methionine. Radioactivity was detected in the fractions corresponding to m^{5}Cyt suggesting the presence of this methylated base. m^{6}Ade could not be detected by this method (Table I) nor when labelling of DNA was performed with [2-^{3}H]adenosine (data not shown).

Fig. 1A shows an HPLC analysis of canonical and methylated bases in hydrolyzed *T. cruzi* $_N$DNA. A peak

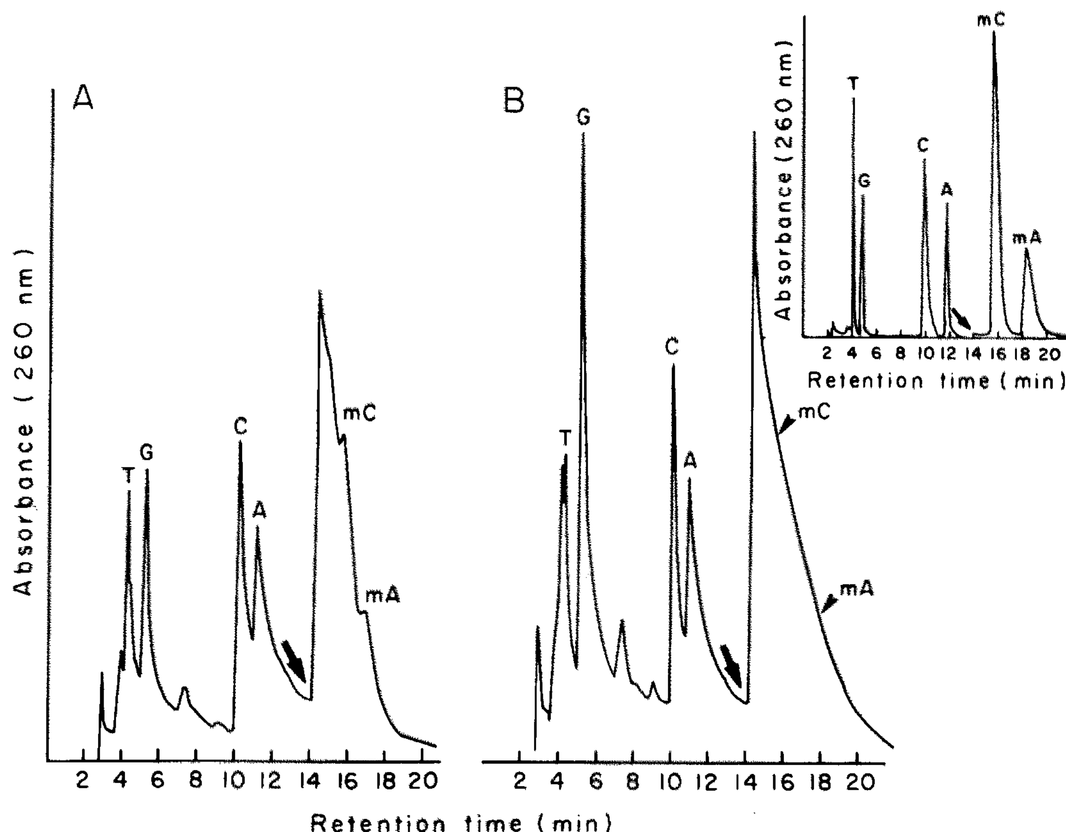

Fig. 1. Purine and pyrimidine bases from a hydrolyzed *T. cruzi* $_N$DNA were analyzed by HPLC at 1 ml/min using a 20 min linear gradient of 12% methanol/20 mM ammonium formate to 12% methanol/40 mM ammonium formate, pH 4.0. (A) Non-treated cultures. (B) Cultures treated daily, for 14 days, with 10^{-6} M of 5-aza-C. The column was pre-run with the canonical and methylated standard bases (insert). Arrows, 1/16 change in the scale, at 14 min.

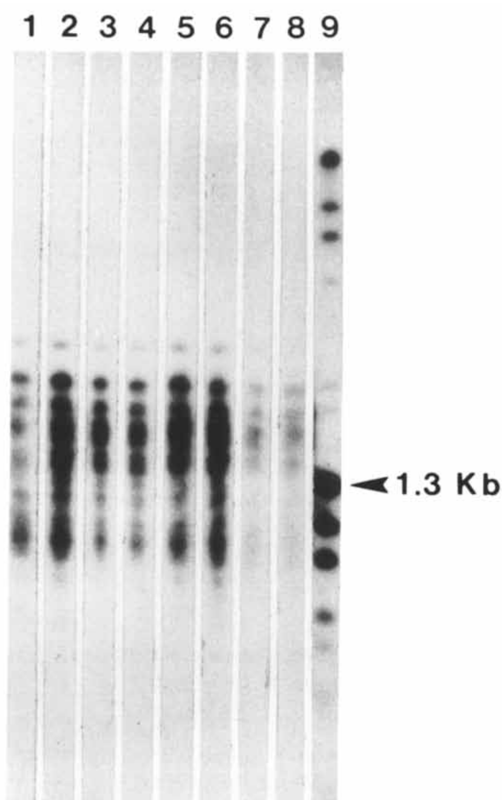

Fig.2. DNA of *T. cruzi* epimastigotes Tul 0 Chile, 14 days (lanes 1, 2) and 5 days (lanes 3, 4) of culture. DNA of *T. cruzi* RA epimastigotes (lanes 5, 6) and trypomastigotes (lanes 7,8). DNA from lambda and ϕX174 phages digested with *Hin*dIII and with *Hae*III, respectively (lane 9). Digestion with *Msp*I: 1, 3, 5 and 7. Digestion with *Hpa*II: 2, 4, 6 and 8. Hybridized with ^{32}P-labelled clone 13 [20].

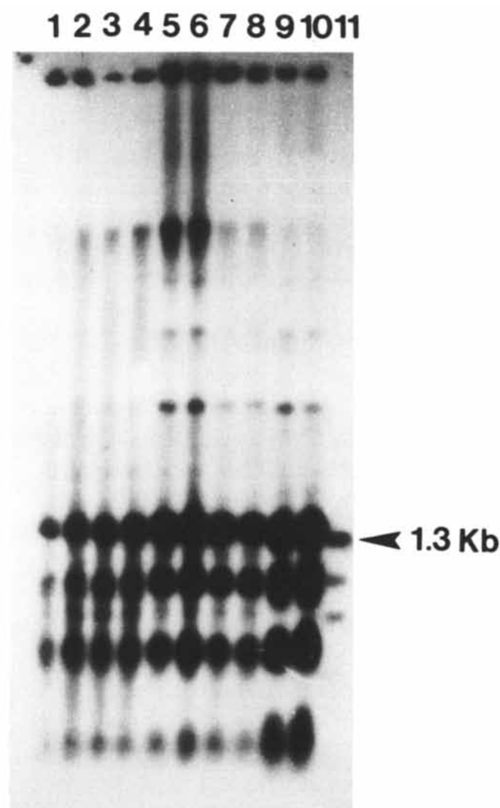

Fig. 3. DNA of *T. cruzi* epimastigotes Tul 0 Chile, 14 days (lanes 1, 2) and 5 days (lanes 3, 4) of culture. DNA of *T. cruzi* RA epimastigotes (lanes 5, 6) and trypomastigotes (lanes 7, 8). DNA from *T. cruzi* epimastigotes Tul 0 Arg (lanes 9, 10). DNA from lambda and from ϕX174 phages digested with *Hin*dIII and with *Hae*III, respectively (lane 11). Digestion with *Msp*I: 1, 3, 5, 7 and 9. Digestion with *Hpa*II: 2, 4, 6, 8 and 10. Hybridized with ^{32}P-labelled total *T. cruzi* DNA strain Tul 0.

in the expected elution position of m^{5}Cyt is shown; another short peak near the elution position of m^{6}Ade is also evident. Both peaks are absent after treatment of the cells with 5-aza-C (Fig. 1B). The apparent sharp increase in absorbance prior to m^{5}Cyt results from a change in the sensitivity of the register, which is necessary to magnify the signals corresponding to methylated bases. These results confirm the presence of m^{5}Cyt, and suggest that m^{6}Ade or another similar modified base is also present in *T. cruzi* DNA.

In Fig. 2, the restriction pattern for *Msp*I and *Hpa*II of *T. cruzi* $_N$DNA obtained from day 14 (lanes 1, 2) and day 5 (lanes 3, 4) of culture are shown. Simultaneously, restriction fragments produced by the same enzymes with DNA from epimastigotes (lanes 5, 6) and trypomastigotes (lanes 7, 8) from the strain RA are also shown. Restriction bands were hybridized to ^{32}P-labelled DNA from clone 13 [20].

No evident differences in the pattern of the restriction fragments were observed, in resting (14 days) or growing (5 days) and trypomastigotes (Go cells). Similar results were obtained when clones 2, 7 and 30 ([20], data not shown) or when total $_N$DNA from Tul 0 (Fig. 3) were used as probes.

These data strongly suggest that the sequences CCGG are not methylated in $_N$DNA from the *T. cruzi* strains tested and under the different conditions of proliferation and cell differentiation assayed.

4. DISCUSSION

The data presented here constitute the first clear demonstration that m^{5}Cyt occurs in $_N$DNA of *T. cruzi*. In a previous report, Riou and Pautrizel [21] reported the absence of modified bases in *T. cruzi* DNA hydrolyzed with perchloric acid for 1 h at 100°C and analyzed by cellulose thin-layer chromatography. This technique permits the detection of bases when their concentration is high enough to be detected by ultraviolet light. Considering the usual low concentration of modified bases in $_N$DNA of higher eukaryotes, with the exception of plants, it is not surprising that m^{5}Cyt was not found by cellulose thin-layer chromatography. In fact, when we analyzed a perchloric acid-hydrolyzed *T. cruzi* $_N$DNA by paper chromatography, we were not able to detect any spot

co-migrating with modified bases (data not shown). In order to detect these bases, it was necessary to label the DNA with appropriate precursors, as was previously described for *Tetrahymena* [4,5]. Finally, the use of a technique with a high sensitivity such as HPLC permitted the clear demonstration of modified bases in $_N$DNA of *T. cruzi* (see Fig. 1). The amount of m^{5}Cyt and of m^{6}Ade should be over 0.1 mol% (one methylated base for each 1000 nucleotides), which is the resolution limit of the HPLC technique [22].

Although m^{5}Cyt residues in DNA of eukaryotes organisms are frequently present in CCGG sequences [3], apparently this is not the case in *T. cruzi* DNA. One possibility is that m^{5}Cyt is present in a sequence such as XCGX, which would not be recognized by the *Hpa*II/*Msp*I system. Another possibility is that m^{5}Cyt is located in other different sequences such as CA, CT or CC [2]. It is also possible that only a few of the CCGG sequences are methylated. In this case, the use of the system of restriction endonucleases *Hpa*II and *Msp*I would not be sensitive enough to detect the presence of this modified base. In such a case, our results would indicate that widespread methylation does not occur in CCGG sequences of *T. cruzi* DNA. Alternatively, the frequency of this sequence in total DNA may be low. Furthermore, this method probes only for a subset of the CpG sequences [23]. In any case, it should be taken into account that methylation in a few bases, or even in one, is sufficient for changing the transcription activity of a gene [24].

Considering that our results point to the presence of m^{5}Cyt in *T. cruzi*, the cytosine analog 5-aza-C should produce $_N$DNA hypomethylation. We have found that this is the case, indicating that m^{5}Cyt is indeed present in $_N$DNA of this parasite.

Interestingly, m^{6}Ade or a related modified base seems also to be present in *T. cruzi* $_N$DNA. This modified base was previously described in other unicellular eukaryotes such as *Tetrahymena* [4,5], *P. aurelia* [25] and *C. reinhardi* [26]. Surprisingly, this modified base was absent when the cells were previously treated with 5-aza-C. Considering that this drug induces DNA hypomethylation by its incorporation into DNA instead of cytosine and by inhibition of methylases [11], this result suggests that both bases are methylated by the same enzyme, or that both enzymes are inhibited by the drug.

Modified bases have not been found heretofore in DNA from African trypanosomes [6–9]. As it is widely known, African trypanosomes present a mechanism for evasion of the immunological response of the host that is completely different from the strategy followed for the same purpose by American trypanosomes [1]. Interestingly enough, different mechanisms of regulation of gene expression may operate in these parasites. Thus, it is not fully unexpected to find DNA methylation in *T. cruzi*, while this DNA modification may not be present in African trypanosomes.

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