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African Trypanosomes Contain 5-Methylcytosine in Nuclear DNA▽†

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It is currently unclear if there are modified DNA bases in *Trypanosoma brucei* other than J-base. We identify herein a cytosine-5 DNA methyltransferase gene and report the presence and location of 5-methylcytosine in genomic DNA. Our data demonstrate that African trypanosomes contain a functional cytosine DNA methylation pathway.

Experiments from the early 1980s demonstrated that inactive *Trypanosoma brucei* variant surface glycoprotein (VSG) genes were resistant to digestion by certain restriction enzymes, suggesting the presence of modified DNA bases (2, 18). Searches for the presence of modified DNA bases in *T. brucei* uncovered J-base and its precursor, 5-hydroxymethyluracil (11, 12). It generally has been assumed that no other modified DNA bases exist in *T. brucei.*

Since the modified DNA base 5-methylcytosine (5MC) is widespread in prokaryotes and higher eukaryotes, we searched for genes capable of encoding a cytosine-5 DNA methyltransferase (C5-DNA MTase) in *T. brucei*. TBLASTN was used to search the *T. brucei* TREU927 nuclear genome sequence, using the *Escherichia coli* Dcm C5-DNA MTase protein as a query (4, 16). A significant match ($E = 1.2 \times 10^{-20}$) was found on *T. brucei* chromosome 3, and we named the locus the TbDMT gene. The TbDMT gene codes for a protein with a predicted molecular mass of 69 kDa, and it is now listed as a putative C5-DNA MTase in GeneDB (www.genedb.org). No other *T. brucei* C5-DNA MTase homologs were identified by BLAST analyses with the Dcm protein or other queries, suggesting but not proving that *T. brucei* has a single C5-DNA MTase. The alignment of the predicted TbDMT protein sequence with experimentally validated prokaryotic C5-DNA MTases indicates that TbDMT contains the 10 conserved domains found in all C5-DNA MTases, including the catalytic cysteine residue of domain IV (Fig. 1) (19). The predicted TbDMT protein is more homologous to prokaryotic enzymes than to eukaryotic enzymes with respect to the 10 conserved domains (data not shown). However, TbDMT contains an N-terminal extension that is longer than that of most prokaryotic enzymes, which is a characteristic of the human DNMT1 and DNMT3A/B enzymes (10). Quantitative PCR analysis of RNA from *T. brucei* bloodstream-form (BF) and procyclic-form (PF) parasites indicates that the TbDMT gene is expressed in both stages. BF parasites have 2.3 (±0.3, which is one standard deviation) times more TbDMT RNA using β-tubulin as a loading control and 1.5 (±0.2) times more TbDMT RNA using 18S rRNA as a loading control, indicating that there is little stage-specific regulation.

The expression of TbDMT in both PF and BF parasites warranted the examination of DNA from these stages for the presence of 5MC. We began by using a blotting assay with a monoclonal antibody against 5MC (Fig. 2) (9, 15, 17). DNAs from *T. brucei* and control organisms were treated with sodium hydroxide to remove RNA, spotted onto a nitrocellulose membrane, fixed via baking, and incubated overnight with a 1:5,000 dilution of anti-5MC antibody (Calbiochem). Antibody binding was detected by chemiluminescence after incubation with a 1:10,000 dilution of peroxidase-labeled anti-mouse secondary antibody. The antibody reacted strongly with *Homo sapiens* DNA from placental tissue and *E. coli* JM109 DNA (*dcm*$^+$), as they contain 5MC (23, 25). The antibody did not react with DNAs from *Saccharomyces cerevisiae* or *E. coli* ER2925 DNA (*dcm* mutant), as these DNAs lack 5MC (20). These control experiments clearly demonstrate the specificity of the antibody for 5MC. *T. brucei* PF and BF DNAs both were positive for the presence of 5MC in this assay. The signal intensity for the *T. brucei* samples was less than that of human and *E. coli* JM109 DNA in all experiments. The *T. brucei* DNA signal in this assay is not due to residual RNA, because the DNAs were treated with sodium hydroxide prior to spotting, and purified, DNase-treated *T. brucei* RNA does not react with the antibody under these conditions (data not shown). These data indicate that 5MC is found in *T. brucei* genomic DNA.

The presence of 5MC in *T. brucei* genomic DNA was confirmed using liquid chromatography-electrospray ionization tandem mass spectrometry analysis according to Song et al. (23). DNA (1 μg) was hydrolyzed to dephosphorylated deoxynucleosides, separated by liquid chromatography, and ionized. Tandem mass spectrometry was utilized to detect the mass/charge ratio of the molecular ion (241.2 atomic mass units) and product ion (126.3 atomic mass units) of 5-methyl-2′-deoxycytidine (5MdC) (Fig. 3). The signal intensity for

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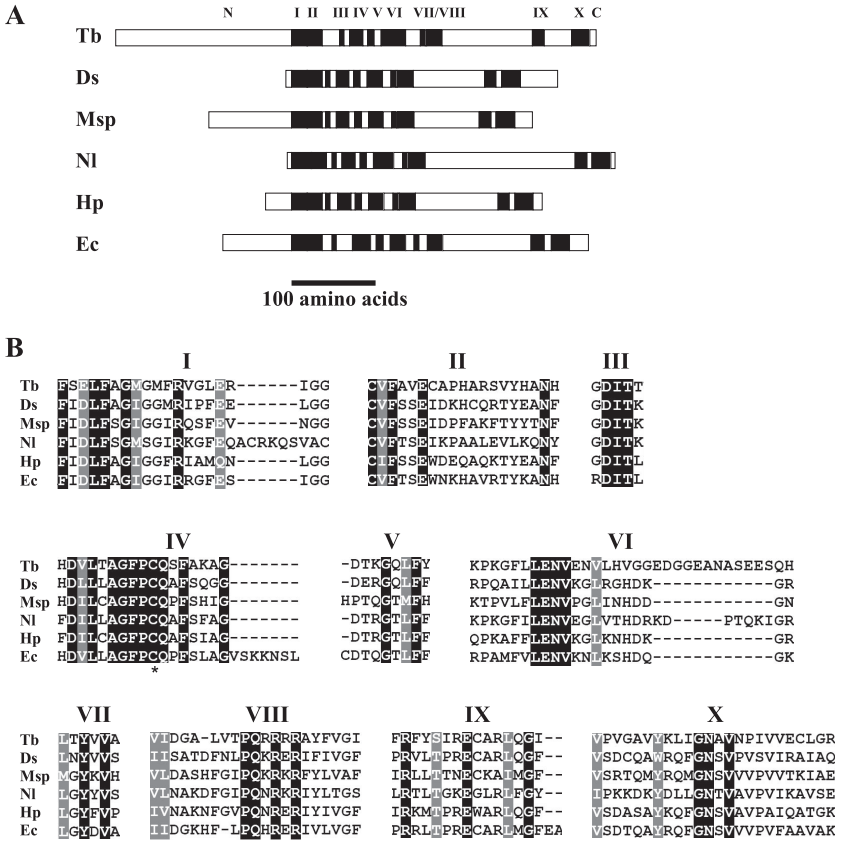

FIG. 1. Multiple-sequence alignment of TbDMT with bacterial C5-DNA MTases. Proteins homologous to TbDMT in the Swiss-Prot database were identified using BLASTP. A multiple-sequence alignment was created with T-Coffee. The sequences (abbreviations and Swiss-Prot/GenBank accession numbers are listed in parentheses) are from *Dactylococcopsis salina* (Da; P50185), a *Moraxella* species (Msp; P11408), *Neisseria lactamica* (Nl; P50182), *Haemophilus parainfluenza* (Hp; P15446), and *Escherichia coli* (Ec; P0AED9), all of which are experimentally validated C5-DNA MTases. Tb, *T. brucei.* (A) Domain structure of aligned C5-DNA MTases. Roman numerals above black boxes represent the 10 conserved domains found in all C5-DNA MTases. N represents nonconserved N-terminal extensions, and C represents short, nonconserved C-terminal extensions. (B) Sequence of the 10 conserved domains of the C5-DNA MTases (indicated by Roman numerals). Black shading indicates residues that are identical in all sequences, and gray shading indicates residues that are structurally similar in all sequences. The putative catalytic cysteine residue in domain IV is indicated with an asterisk.

5MdC was divided by the intensity of 2′-deoxyguanosine and compared to a standard curve of the same deoxynucleosides. *E. coli* strain BW25113, a wild-type strain (1), contains 0.99% (±0.33%, which is one standard deviation) 5MdC, which is consistent with previous studies of other *E. coli* strains (25).

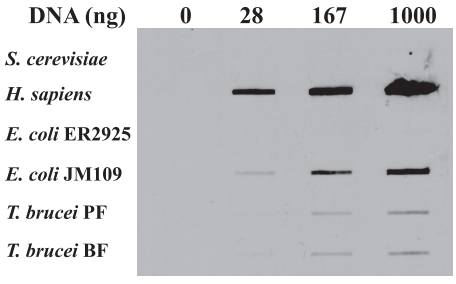

FIG. 2. Antibody-mediated detection of 5MC in *T. brucei* DNA. Various amounts of genomic DNA from *S. cerevisiae*, *H. sapiens*, *E. coli* strain ER2925 (*dcm* knockout), *E. coli* strain JM109 (*dcm*$^+$), *T. brucei* PF parasites, and *T. brucei* BF parasites were spotted onto a nitrocellulose membrane. After fixation, the filter was probed with a monoclonal antibody against 5MC. Bound antibody was detected using a horseradish peroxidase-conjugated secondary antibody and chemiluminescence.

DNA from *E. coli* JW1944-2, a *dcm* knockout strain (1), displays no detectable 5MdC signal above background. 5MdC was detected in *T. brucei* PF and BF DNA using this strategy, confirming the detection of 5MC using the blotting assay. The levels of 5MdC in these samples are low and made precise quantification difficult. However, the signal for the two *T. brucei* samples is at least 0.01% 5MdC, as the limit of detection of the assay is 0.01% 5MdC. Thus, there is a minimum of 1 5MC for every 10,000 cytosines in the *T. brucei* genome. The mass/charge ratio of the molecular ion and product ion of the modified deoxynucleosides clearly indicates that the base is 5MdC and not 5-methylcytidine (an RNA base) or another modified base that previously has gone undetected.

To identify the location of 5MC within the *T. brucei* genome, an immunoprecipitation strategy was utilized (9, 21). *T. brucei* PF and BF DNAs (2 μg) were digested with DpnII, and linkers were added to facilitate PCR amplification. DNA was denatured and immunoprecipitated with either an immunoglobulin G1 (IgG1) monoclonal antibody against 5MC or an IgG1 isotype control antibody. DNA-antibody complexes were captured using magnetic beads coated with sheep anti-mouse IgG (Invitrogen), eluted via proteinase K treatment, and briefly

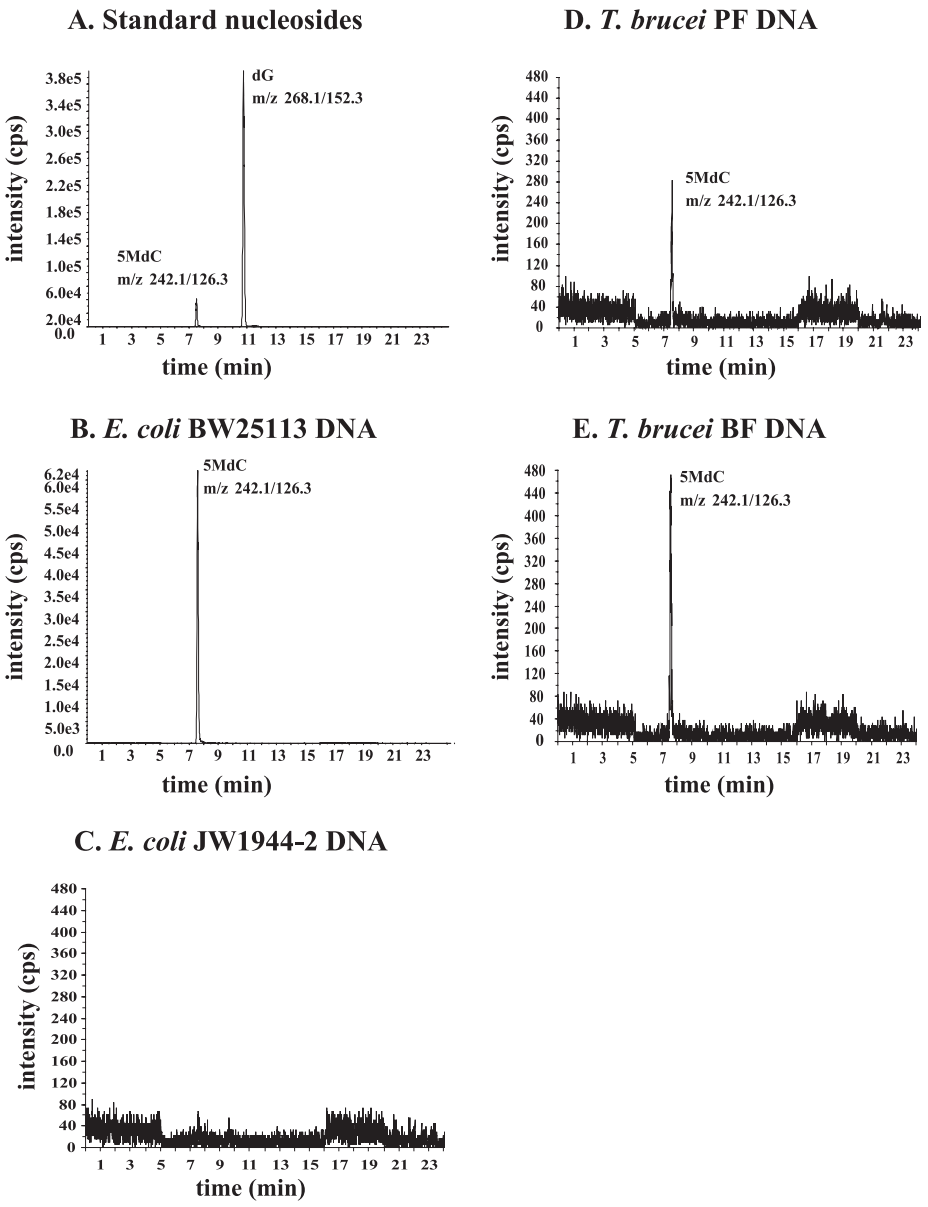

FIG. 3. Detection of 5MdC in *T. brucei* DNA using liquid chromatography-electrospray ionization tandem mass spectrometry. (A) Chromatograph of a standard containing 5′-methyl-2′-deoxycytidine (5MdC) and 2′-deoxyguanosine (dG) at a 1:10 ratio. Multiple-reaction monitoring was used to detect molecular ion/product ions for 5MdC (*m*/*z* 242.1/126.3) and dG (*m*/*z* 268.1/152.3). Chromatographs of hydrolyzed (B) *E. coli* BW25113 DNA (*dcm*$^+$), (C) *E. coli* JW1944-2 DNA (*dcm* knockout), (D) *T. brucei* PF DNA, and (E) *T. brucei* BF DNA. The molecular ion/product ion data for dG is not shown. The *y* axis of each graph has a scale appropriate for the sample.

amplified using 15 cycles of PCR. A robust PCR signal was obtained from the anti-5MC immunoprecipitates, whereas no PCR signals were detected from the IgG1 isotype control immunoprecipitates or from reactions with unmodified *T. brucei* DNA (see Fig. S1 in the supplemental material).

PCR products from the anti-5MC immunoprecipitates were inserted into the pGEM-T Easy plasmid (Promega). Plasmids were isolated from randomly selected colonies and analyzed by DNA sequencing. BLASTN analysis was used to search both the *T. brucei* database at GeneDB and the nonredundant nucleotide collection (nr/nt) of the National Center for Biotechnology Information (Table 1). All immunoprecipitated DNAs were bona fide *T. brucei* DNAs, as the lowest E values were matches to known *T. brucei* sequences. All immunoprecipitated DNAs were from the nuclear *T. brucei* genome. No sequences from mitochondrial maxicircles or minicircles were immunoprecipitated. Several different nuclear loci were present in the immunoprecipitate, which is consistent with the heterogeneous PCR products produced. This demonstrates that 5MC is not restricted to a single locus in *T. brucei*.

Nonetheless, it is possible that there are clusters of 5MC, as some of the immunoprecipitated DNA sequences correspond to the same gene category. For example, retrotransposon hot spot (RHS) loci were highly represented in this analysis, as

TABLE 1. *T. brucei* DNAs containing 5MC

Locus name or description	DNA	Locus ID[a]	E value
40S ribosomal protein S14	PF	Tb11.0390	1.10E-35
ATP-dependent DEAD/H DNA helicase *recQ*	BF	Tb927.6.3580	8.60E-102
Chaperone protein DNAJ	PF	Tb11.01.8480	7.30E-188
Chaperone protein DNAJ	PF	Tb10.70.0170	3.80E-85
Cytochrome oxidase subunit X	PF	Tb11.01.4702	4.30E-13
Developmentally regulated GTP-binding protein	PF	Tb09.211.0720	1.00E-86
DNA-directed RNA polymerase II, subunit 2	PF	Tb927.4.3810	1.60E-42
Electron transfer flavoprotein-ubiquinone oxidoreductase	PF	Tb927.8.1240	6.80E-46
Elongation factor 1α	PF	Tb10.70.5650	4.00E-93
Elongation factor 2	PF	Tb10.70.2650	4.40E-45
Eukaryotic translation initiation factor 3, subunit 7-like protein	BF	Tb927.6.4370	3.70E-143
Expression site-associated gene (pseudogene)	BF	Tb927.3.2530	1.80E-22
GSS (unannotated)[b]	BF	AL454589 (GenBank)	1.30E-145
GSS (unannotated)[b]	BF	AQ642500 (GenBank)	6.00E-46
Hypothetical protein	PF	Tb10.v4.0082	3.90E-07
Hypothetical protein	BF	Tb927.5.297b	4.90E-54
Hypothetical protein, conserved	PF	Tb927.7.2560	1.30E-93
Hypothetical protein, conserved	PF	Tb927.8.7360	3.60E-69
Hypothetical protein, conserved	PF	Tb927.8.2660	1.40E-65
Hypothetical protein, conserved	PF	Tb927.7.6130	1.60E-23
Hypothetical protein, conserved	PF	Tb11.01.2540	3.60E-44
Hypothetical protein, conserved	PF	Tb09.160.5570	5.50E-46
Hypothetical protein, conserved	PF	Tb10.26.0910	5.30E-124
Hypothetical protein, conserved	PF	Tb927.1.3910	1.10E-87
Hypothetical protein, conserved	PF	Tb09.211.2550	2.00E-72
Hypothetical protein, conserved	PF	Tb927.8.3230	8.10E-18
Hypothetical protein, conserved	BF	Tb927.5.2770	2.10E-63
Hypothetical protein, conserved	BF	Tb09.160.4870	6.40E-209
Hypothetical protein, conserved	BF	Tb927.4.1890	1.30E-67
Hypothetical protein, conserved	BF	Tb927.4.2470	1.10E-123
Hypothetical protein, conserved	BF	Tb11.02.2520	2.70E-21
Hypothetical protein, conserved	BF	Tb10.406.0620	1.30E-76
Hypothetical protein, conserved	BF	Tb927.6.410	1.80E-183
Hypothetical protein, conserved	BF	Tb927.2.2410	2.10E-132
Hypothetical protein, conserved	BF	Tb11.55.0022	3.30E-58
Hypothetical protein, conserved	BF	Tb927.8.5490	7.10E-134
Hypothetical protein, conserved	BF	Tb927.3.3830	1.20E-136
Intergenic sequence[c]	PF	Between Tb11.01.3790 and Tb11.01.3800	7.00E-74
Intergenic sequence[c,d]	PF	Between Tb11.14.0010 and Tb11.14.0011	2.60E-35
Intergenic sequence[c]	BF	Between Tb927.2.2590 and Tb927.2.2650	3.60E-94
Intergenic sequence[c]	BF	Between Tb09.160.0790 and Tb09.v1.0080	7.60E-16
Intergenic sequence[c]	BF	Between Tb09.160.2360 and Tb09.160.2370	1.20E-56
Kinesin	PF	Tb927.7.3000	2.70E-183
Kinesin	BF	Tb927.5.2410	1.70E-159
Minichromosome maintenance complex subunit	PF	Tb11.02.5730	1.50E-43
Procyclin-associated gene 2 protein	BF	Tb10.70.1300	1.70E-33
Proteasome regulatory ATPase subunit 3	PF	Tb927.6.1090	6.40E-132
Protein kinase	BF	Tb927.5.3320	8.40E-115
Receptor-type adenylate cyclase GRESAG 4	BF	Tb927.4.4450	1.40E-127
RHS protein	PF	Tb927.2.450	1.40E-144
RHS protein	BF	Tb927.2.370	3.10E-155
RHS protein	BF	Tb927.1.70	8.70E-183
RHS pseudogene	PF	Tb927.1.90	1.70E-69
RHS pseudogene	BF	Tb11.1800	1.50E-103
RHS pseudogene	BF	Tb927.1.430	2.50E-81
RNA-binding protein	PF	Tb927.8.4170	1.90E-80
RuvB-like DNA helicase	BF	Tb927.4.1270	7.60E-132
Serine/threonine protein kinase	BF	Tb09.160.1090	1.10E-140
SLACS retrotransposable element (part)	PF	Tb09.211.5010	3.00E-122
VSG pseudogene	PF	Tb11.21.0002	3.10E-11
VSG pseudogene	PF	Tb927.6.5210	3.00E-76
VSG pseudogene	PF	Tb927.3.170	1.00E-60
VSG pseudogene	BF	Tb11.16.0004	2.40E-163
Vesicular transport protein (CDC48 homologue)	PF	Tb11.55.0014	1.40E-75
Zeta tubulin	BF	Tb927.1.1150	1.30E-56

[a] The sequence overlaps with the open reading frame; the GeneDB locus identity (ID) is provided unless otherwise indicated.
[b] Sequences found in the GSS (genome survey sequence) database but not the assembled genome.
[c] Found between two protein-coding genes.
[d] Found between two VSG pseudogenes.

6/65 sequences correspond to these loci. RHS genes form a family of approximately 280 members and code for nuclear proteins of unknown function (5). Approximately ~60% of all RHS genes are pseudogenes that often contain the retrotransposons Ingi or RIME (5, 6). RHS expression is repressed by the RNA interference machinery (7). 5MC at RHS loci may be used in conjunction with or in addition to RNA interference to repress the transcription of unwanted RHS pseudogenes and Ingi/RIME retrotransposons, as 5MC has this function in many other organisms (26). In support of the model of retrotransposon silencing, one sequence representing the site-specific retrotransposon SLACS (splice leader associated conserved sequence) also was immunoprecipitated.

VSG loci also were highly represented, as 4/65 sequences represent VSG pseudogenes and 1/65 sequences represents an intergenic region between two VSG pseudogenes. The *T. brucei* TREU927 genome contains >1,200 VSG genes, and the majority are pseudogenes (3). Evidence suggests that one VSG is expressed at a time in BF parasites from 1 of 20 bloodstream expression (bES) sites, and the expressed VSG can be switched by multiple mechanisms (24). The remaining bES genes are transcriptionally repressed in each stage. In contrast, no VSGs are expressed in PF parasites (24). The mechanism of VSG transcriptional silencing has remained a key question. Recent evidence suggests that proteins that modify chromatin and its associated histones, TbISW1 and DOT1B, play a role in bES silencing (8, 13). Thus, it is possible that 5MC is used in addition to or in conjunction with these proteins to silence the transcription of some or all bES genes and/or pseudogenes in these stages to ensure that only one functional VSG is expressed at a time. This hypothesis is based on the strong correlation of 5MC and heterochromatin-induced transcriptional repression in other organisms (22). Roles for 5MC other than transcriptional repression in *T. brucei* require consideration as well, since RHS and VSG loci were not the only methylated loci identified, and the transcriptional regulation of housekeeping genes in *T. brucei* is not thought to exist. Since modified bases in other organisms affect biological processes including genome stability, DNA replication, and DNA repair (14), it is possible that 5MC could do the same in *T. brucei*. In the future, we aim to identify the locations of 5MC in the *T. brucei* genome at the nucleotide level, elucidate the function of 5MC, and determine the role of the TbDMT protein in this pathway.

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