The TbMTr1 Spliced Leader RNA Cap 1 2′-O-Ribose Methyltransferase from *Trypanosoma brucei* Acts with Substrate Specificity

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In metazoan cap 1 (m7GpppNmp-RNA) is linked to higher levels of translation; however, the enzyme responsible remains unidentified. We have validated the first eukaryotic encoded cap 1 2′-O-ribose methyltransferase, TbMTr1, a member of a conserved family that modifies the first transcribed nucleotide of spliced leader and U1 small nuclear RNAs in the kinetoplastid protozoan *Trypanosoma brucei*. In addition to cap 0 (m7GpppNp-RNA), mRNA in these parasites has ribose methylations on the first four nucleotides with base methylations on the first and fourth (m7Gpppm6,6AmpAmpCmpm3Ump-SL RNA) conveyed via trans-splicing of a universal spliced leader. The function of this cap 4 is unclear. Spliced leader is the majority RNA polymerase II transcript; the RNA polymerase III-transcribed U1 small nuclear RNA has the same first four nucleotides as spliced leader, but it receives an m2,2,7G cap with hypermethylation at position one only (m2,2,7Gpppm6,6AmpAmpCmpm3Ump-U1 snRNA). Here we examine the biochemical properties of recombinant TbMTr1. Active over a pH range of 6.0 to 9.5, TbMTr1 is sensitive to Mg2+ substituted to H1034 solely to indicate this fact. Positions Lys95-Lys259-Glu285 constitute the conserved catalytic core. A guanosine cap on RNA independent of its N7 methylation status is required for substrate recognition, but an m2,2,7G-cap is not recognized. TbMTr1 preferentially recognizes a leader 5′ sequence, as reflected by a preference for A at position 1 and modulation of activity for substrates with base changes at positions 2 and 3. With similarities to human cap 1 methyltransferase activity, TbMTr1 is an excellent model for higher eukaryotic cap 1 methyltransferases and the consequences of cap 1 modification.

Most mature eukaryotic and viral mRNAs possess a 5′ cap that consists of an inverted guanosine methylated at position N7 linked to the first transcribed RNA nucleotide by a unique 5′-3′ triphosphate bond (m7GpppN). This cap 0 is necessary for mRNA stability and increased translational efficiency (1). The enzymes involved in the capping reaction are conserved. The three enzymatic activities catalyzing the three-step capping process, RNA triphosphatase, RNA guanylyltransferase, and RNA methyltransferase (MTase), have been identified and characterized in detail. Further modification of the cap is found in higher eukaryotes including mRNAs from insects, vertebrates, and associated animal viruses with additional ribose 2′-O-ribo-methylations of the first (cap 1) and second (cap 2) nucleosides (2). Despite their widespread presence, little is known about the functional significance of mRNA cap 2′-O-ribose methylations in the eukaryotes, including their mechanism of formation, beyond the partial purification of two separate cap 1 and cap 2 2′-O-ribose MTase activities from HeLa cell extracts (3–5).

Nature’s most hypermethylated cap is present on the spliced leader (SL) RNA of the Kinetoplastidae, a family containing a number of medically important unicellular parasites including *Trypanosoma brucei*, *Leishmania* spp., and *Trypanosoma cruzi*. In addition to the standard cap 0, it has seven methylations collectively referred to as cap 4. The SL RNA cap 4 is composed of 2′-O-ribose methylations on the first four transcribed nucleosides with additional base methylations at the first and fourth positions (m7Gpppm6,6AmpAmpCmpm3Ump-SL RNA) (6). The biogenesis of mRNA in kinetoplastid parasites involves post-transcriptional processing of long polycistronic precursors into individual protein-coding mRNAs by the two physically coupled events of 5′ trans-splicing and 3′ polyadenylation (7, 8). Trans-Splicing also serves as a trans-capping reaction by attaching the 39-nucleotide SL with 5′-cap 4 to all mature mRNA.

The abbreviations used are: MTase, methyltransferase; AcNPV, *Autographa californica* nucleopolyhedrosis virus; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosyl-L-methionine; rTbMTr1, recombinant TbMTr1; SL spliced leader; snRNA, small nuclear RNA; TAP, tobacco acid pyrophosphatase; TMG, trimethylguanosine; WNV, West Nile flavivirus; aa, amino acid.
The role(s) of the kinetoplastid cap 4 has yet to be determined. Studies using methylation inhibitors suggested that complete cap 4 was necessary for efficient use of the SL RNA in pre-mRNA trans-splicing (9, 10). However, mutated and undermethylated SL are trans-spliced in vivo (11–13). An alternate role for mature cap 4 could be in controlling translational efficiency, as mRNA bearing exon-mutated undermethylated cap 4 structures showed decreased polysomal association (12). Three genes encoding proteins responsible for ribose 2'-O-ribose methylations at positions 1 (TbMTr1), 2 (TbMTr2), 3, and possibly 4 (TbMTr3) of the SL RNA have been identified (14–18). RNA interference-mediated knockdown or allelic knock-out of the individual genes, as well as simultaneous knockdown of TbMTr2 and TbMTr3, resulted in undermethylated SL competent for trans-splicing with no significant effect on cell growth. The rationale for maintenance of this elaborate 5' structure is still unclear, although the widespread conservation of the cap 4 throughout the family indicates a selected benefit. Likewise, SL sequence conservation is implicated primarily in the process of cap 4 acquisition and interaction with the translation machinery.

Cap 1-specific 2'-O-ribose MTases have been studied extensively in viral systems. Vaccinia virus VP39, the best characterized among this group, serves dual functions of an S-adenosylmethionine (AdoMet)-dependent cap 1 MTase and a non-catalytic processivity subunit of poly(A) polymerase (19). Based on crystal structures, mechanisms of action have been proposed for cap 1 MTase activities residing in domain I of the Reovirus protein λ2 (20) and the N-terminal portion of flavivirus NS5 proteins (21). The orf69 gene in the baculovirus Autographa californica nucleopolyhedrosis virus (AcNPV) also encodes a cap 1 2'-O-ribose MTase (22). Recent characterization of the NS5 protein from West Nile flavivirus (WNV) demonstrated its ability to perform both guanosine N7 and ribose 2'-O-ribosylation that complete the 5' cap (23). Region VI of L protein in vesicular somatitis virus shows similar dual MTase activities with a single AdoMet binding pocket in the protein (24, 25). Both TbMTr1 and TbMTr2 are AdoMet-dependent enzymes (17, 18).

Phylogenetic analyses of the T. brucei cap 1 2'-O-ribose MTase TbMTr1 indicate that it belongs to an unexplored family with baculoviral and higher eukaryotic members, including human, Drosophila, and Caenorhabditis elegans homologs (20). Genetic knock-out resulted in undermethylated substrate SL with a predominantly cap 0 phenotype (18). A significant increase in the cap 0 form of the U1 snRNA was also observed in the TbMTr1 null cells, implying that TbMTr1 is involved in U1 snRNA modification. The link between SL and the U1 snRNA as TbMTr1 substrates is the sequence they share at their 5' ends, which are common for six of the first seven nucleotides. However, in contrast to the m7G cap 0 of SL, mature U1 snRNA carries a trimethylguanosine (TMG) cap (26). To further understand the biochemistry of cap 1 formation, we expressed and purified a recombinant form of TbMTr1. We report the biochemical characterization of the kinetoplastid cap 1 2'-O-ribose MTase TbMTr1, its substrate specificity, and identification of the vital K-D-K-E tetrad critical for AdoMet-dependent MTases.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides**—The oligonucleotides used in this study are shown in supplemental Table 1.

**Cloning and Overexpression of TbMTr1**—Genomic DNA extracted from T. brucei YTAT strain was used as template to amplify the TbMTr1 gene (Tb10.6k15.2610) with Pfu polymerase, using TbMTr1-Fd and TbMTr1-Rv primers. Both primers contain restriction enzyme sites (underlined), such that the forward primer inserted an Ndel site at the ATG start codon and the reverse primer inserted an Xhol site immediately after the stop codon. The 1.2-kb PCR fragment was cloned in the Ndel-Xhol sites of the pET28a expression vector (Novagen) to direct synthesis of the TbMTr1 protein fused to a His6 tag at the N terminus (rTbMTr1).

For overexpression of rTbMTr1, the plasmid was used to transform Escherichia coli BL21(DE3)pLysS (Novagen). Overnight cultures from single colonies were used to inoculate 100 ml of LB media supplemented with 2% ethanol. After growing the cells at 37 °C to a density of 0.6–0.8 OD, the cells were quick-chilled on ice for 5 min and isopropyl β-D-thiogalactopyranoside was added to 0.2 mM final concentration. The cells were further allowed to grow overnight at 22 °C, harvested, and stored at −80 °C until further use.

**Purification of rTbMTr1**—Cells harvested from 100-ml cultures were resuspended in 10 ml of ice-cold buffer A (50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 0.1% Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride) and lysed by sonication. All steps in the purification procedure were carried out at 4 °C. The cell lysate was centrifuged at 12,000 × g for 30 min at 4 °C and the cleared supernatant was loaded onto a 1-ml His-Trap FF column (GE Biosciences) pre-equilibrated with buffer A. After washing the column with 20 ml of buffer B (50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 0.1% Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride) and lysed by sonication, the His-tagged proteins were eluted from the column with 15 ml of buffer C containing 100 mM NaCl, 0.1% Triton X-100, 300 mM imidazole, and 0.1 mM phenylmethylsulfonyl fluoride. The eluted fractions were pooled and dialyzed against buffer C (50 mM Tris-HCl, pH 7.9, 10% glycerol, 0.5 mM EDTA, 50 mM NaCl, and 1 mM dithiothreitol) with three changes every 2 h. The dialyzed sample was further loaded onto a 2-ml phosphocellulose P11 column (Whatman) pre-equilibrated with buffer C. The column was washed with 20 ml of buffer C containing 100 mM NaCl. rTbMTr1 protein was finally eluted from the column with buffer D (50 mM Tris-HCl, pH 7.9, 10% glycerol, 0.5 mM EDTA, 350 mM NaCl, and 1 mM dithiothreitol). Fractions containing purified rTbMTr1 protein were pooled and dialyzed against buffer C with three changes at 2-h intervals, aliquoted, and stored at −80 °C. All purified proteins were quantified using the Pierce BCA kit with bovine serum albumin as standard.

**Preparation of Capped RNA Substrates**—To generate RNA transcripts representing the T. brucei SL RNA sequence, the 150-nucleotide gene was amplified from T. brucei genomic DNA using Pfu polymerase using AgCU-SL:FD and SL:RV as forward and reverse primers (supplemental Table 1). Because the SL RNA starts with an adenine, the forward primer was designed to include a T7 bacteriophage class II d2.5 promoter (27) that is efficient in initiating transcription with an adeno-
sine residue (bold). DNA templates for transcribing mutated SL RNAs with substitutions in the first three nucleotides (lower-case) were prepared similarly by PCR amplification using mutation specific forward primer and SLRV as reverse primer. The PCR products were gel purified and then used as template for transcription reactions performed with T7 Megashortscript Kit (Ambion).

Capping of the transcripts was carried out using [α-32P]GTP (800 Ci/mmol; PerkinElmer Life Science) and vaccinia virus guanylyltransferase enzyme (Ambion) using the manufacturer’s recommendation. For generating 32P-labeled G-capped transcripts (G*pppApGp-SL; * = 32P), AdoMet (New England Biolabs) was left out of the reaction mixture, but was included at 1 mM final concentrations for N7-methyl G-capped transcripts (m7G*pppApGp-SL). Following the reaction the 32P-G cap-labeled RNA transcripts were extracted with phenol/chloroform and passed three times through Sephadex G-25 spin columns to remove any free radionucleotides.

MTase Assay—MTase assays were usually performed in a 20-μl volume by incubating 10 pmol of purified rTbMTr1 protein with 330 fmol of 32P-G cap-labeled m7G*pppApGp-SL RNA as substrate in the reaction mixture containing 25 mM HEPEs pH 8.0, 2 mM dithiothreitol, 50 μM AdoMet, and 2 units of SUPERase-IN (Ambion). Unless mentioned otherwise, incubations were carried out at 28 °C for 60 min, following which RNA was extracted from the reactions by phenol/chloroform treatment, and precipitated with 0.5 μg of yeast tRNA as carrier in ethanol. RNA samples were resuspended in 8 μl of water, and sodium acetate, pH 5.2, was added to a final concentration of 50 mM. Five microliters of the RNA samples were then digested with either 2 μg of nuclease P1 (Sigma) for 2 h or 0.2 units of tobacco acid pyrophosphatase (TAP) for 3 h at 37 °C to liberate the cap structures. Digested samples (1 μl) were spotted on polyethyleneimine-cellulose thin layer chromatography plates (EMD Chemicals), and developed with 0.3 M ammonium sulfate for nuclease P1 digests and 0.45 M ammonium sulfate for TAP digests, respectively. Methylated and unmethylated G caps were spotted along with the samples as markers and their positions were determined by UV shadowing. All MTase assays were performed a minimum of three times. Migration of released cap structures were visualized by PhosphorImager analysis of the TLC plate (GE Healthcare), and identified by comparing with relative positions of the markers. The radiolabeled spots were then quantitated to estimate the extent of cap 1 methylation (m7G*pppAm/m7G*pppAp + m7G*pppAm). For RNase T2 digestion, the radiolabeled RNA was digested in 50 mM ammonium acetate, pH 4.5, and 2 mM EDTA with 40 units/ml RNase T2 at 37 °C for 12 h. Digestion products were resolved on 25% acrylamide, 8 M urea gels and visualized by PhosphorImager (14).

Bioinformatic Analyses of TbMTr1—Data base searches were carried out with PSI-BLAST (28). Cap 1 MTase sequences were aligned using the MUSCLE program (29). The alignment between the cap 1 family and RmMT1 was done using the GeneSilico metaserver (30).

Expression and Purification of Mutated rTbMTr1—Alanine substitution mutations targeting Lys95, Asp207, Lys248, and Glu285 were introduced in the plasmid TbMTr1-pET28a using the QuikChange II Site-directed Mutagenesis Kit (Stratagene) following the manufacturer’s protocol. The resulting plasmids were sequenced to confirm the desired mutations and to exclude the acquisition of unwanted mutations. Plasmids encoding mutated rTbMTr1 proteins were used to transform E. coli BL21(DE3)pLysS cells. All mutated proteins were expressed using the same induction conditions as described for wild-type rTbMTr1, except for the Lys248 mutant that was induced overnight at 16 °C. Purification of the soluble mutated proteins was carried out exactly as described for the wild-type rTbMTr1 protein.

Dinucleotide Competition Assay—Dinucleotide cap analogs were included in the MTase reaction at various mentioned concentrations. GTP, GpppG, m7GpppG, and m7GppppGm7 were obtained from Epitector; m7GpppA, and m2,2′GpppG were synthesized (by J. S. and E. D.) at the Institute of Experimental Physics, Warsaw.

RESULTS

Purification and Activity of rTbMTr1—TbMTr1-null T. brucei show an absence of cap 1 modification on SL RNA and U1 snRNA (18). rTbMTr1 was produced in bacteria to facilitate detailed studies on the enzyme biochemistry, structure, and function. Due to the 5′-sequence identity of the SL RNA and U1 snRNA substrates, we used as substrate an artificial transcript approximating the SL RNA. Position 2 was modified from adenosine to a guanine to facilitate efficient in vitro transcription. A guanine residue at position 2 was utilized efficiently as substrate by TbMTr2 in in vitro studies (17) and minimal effects on rTb-MTr1 activity are demonstrated below.

We cloned TbMTr1 in the E. coli expression vector pET28a and expressed it as a fusion protein with a His6 tag at the N terminus. Following cell lysis, ~80% of the expressed rTbMTr1 was obtained in the soluble fraction. Initial purification was performed on a nickel column and yielded ~90% pure rTb-MTr1. Final purification was achieved by ion-exchange column chromatography on a phosphocellulose P11 column that yielded purified rTbMTr1 apparently free from any contaminating proteins, as verified by SDS-PAGE analysis showing a single polypeptide migrating at 39 kDa (supplemental Fig. 1).

To test MTase activity of the purified protein a 150-nucleotide synthetic RNA with a methylated G-cap (m7G*pppApGp-SL) was used as substrate. Substrate RNA was incubated in a reaction mixture containing AdoMet in the presence and absence of rTbMTr1, following which RNA samples were extracted from the reactions. The 32P-labeled RNA caps were liberated by digestion with nuclease P1, which cleaves capped RNAs into 3′-OH terminated dinucleotide cap structures and 5′-pN-OHs, and separated on a TLC plate (Fig. 1A). Incubation of m7G-capped RNA with rTbMTr1 resulted in the appearance of a faster-migrating species as compared with the m7GpppA marker, indicating that rTbMTr1 can methylate the substrate. Methylation of the base introduces a positive ionic charge so that the mobility of a methylated base is faster compared with the methylated ribose, which alters the mass only and results in a lesser mobility effect. The migration of the m7G-capped rTbMTr1-treated RNA sample indicates a 2′-O-ribose methylation event. The cap structure from mock-treated reactions with
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m7G*pApGp-SL RNA co-migrated with the m7GpppA marker.

2'-O-Ribose methylation of the first transcribed nucleotide was further confirmed by RNase T2 digestion. RNase T2 digestion of RNA sample from rTbMTr1, RNA samples extracted from the reactions were treated with nuclease P1 to release the cap structures and their methylation status was analyzed by TLC on a polyethyleneimine-cellulose sheet. Positions of methylated and unmethylated cap markers are indicated on the right. B, products from reactions with (+) or without (−) the co-factor AdoMet, using m7G*pApGp-SL RNA as substrate were digested with RNase T2 and electrophoresed through a 25% urea-polyacrylamide gel containing 8 M urea.

The protein identified by our in vitro studies as a cap 1 2'-O-ribose MTase was expressed and purified successfully, maintaining the anticipated enzymatic activity. We next sought to determine the biochemical profile of the enzyme to optimize further in vitro analyses.

Activity Parameters of rTbMTr1—rTbMTr1 activity was active over a wide range of pH, with continuous increase in activity from pH 6 through 9.5 (Fig. 2A); a pH of 8.0 was adopted for our standard rTbMTr1 activity assay. Testing of divalent ion dependence revealed that, although Mg2+ is required for activity, the presence of Mg2+ at concentrations above 12.5 mM was found to be inhibitory, but Mn2+ ion did not have any effect even at 25 mM concentrations (Fig. 2B). As shown previously, AdoMet is required as co-factor for functionality. For the assay conditions used, AdoMet dependence was linear between 0.5 and 5 μM and then plateaued (Fig. 2C). The Km for AdoMet was calculated to be 0.8 μM. The activity was inhibited by the presence of S-adenosylhomocysteine (AdoHcy), with about 50% inhibition observed with 250 μM AdoHcy in reactions containing 10 μM AdoMet (Fig. 2D) indicating that the enzyme has a higher affinity for AdoMet. rTbMTr1 activity was unaffected by NaCl concentrations up to 60 mM as well as by the absence of salt supplemented beyond that included in the storage buffer, functionally as low as 0.5 mM NaCl. However, the activity was sensitive to higher salt concentrations with total inhibition at 150 mM (data not shown). The sensitivity of rTbMTr1 to Mg2+ is notable. Mg2+ -mediated inhibition has been shown for several N7-MTase activities, including that of the dual-function WNV NS5 protein. The 2'-O-ribose MTase activity requires 5–10 mM MgCl2, but is inhibited above 12.5 mM, whereas the 5' cap activity is inhibited by any MgCl2 (31). With the basic properties of rTbMTr1 established, we queried the activity for critical features of the protein and RNA substrate.

rTbMTr1 Does Not Methylate the Guanosine Cap—The NS5 protein from WNV performs both the cap 0 guanosine N7 and cap 1 ribose 2'-O-ribose methylation to complete the 5’ cap (23). Region VI of the L protein in vesicular somatitis virus has similar dual MTase activities (24, 25). The AcNPV MTase1 protein and HeLa cap 1 2'-O-ribose MTase utilize both methylated and unmethylated guanosine-capped RNA as substrates. The other trypanosome cap 4 MTases TbMTr2 and TbMTr3 are related to the vaccinia virus VP39 cap 1 2'-O-ribose MTase, and TbMTr2 requires N7 methylation of the guanosine cap for activity. To investigate the relationship between rTbMTr1 and these other cap 1 MTases, we tested its ability to methylate at the N7 position of the guanosine cap. These features are compared in Table 1.

To determine whether rTbMTr1 could methylate a guanosine cap, RNA samples incubated with rTbMTr1 were digested with TAP, hydrolyzing the pyrophosphate bond and liberating m7G*p and G*p from methylated and unmethylated G-capped RNA, respectively. TAP digests of RNA extracted from reactions with the G*KpppApGp-SL RNA substrate treated with or without rTbMTr1 showed identical migrations on TLC plates (Fig. 3A) consistent with that of marker G*p. Likewise, the migration of TAP-treated m7G*KpppApGp-SL samples were identical to that of the m7G marker. Thus, rTbMTr1 does not act upon the G cap.

The ability of rTbMTr1 to use an unmethylated guanosine-capped RNA as substrate was tested by performing the same analysis as presented in Fig. 1A using the non-methylated G*KpppApGp-SL substrate (Fig. 3B). As seen for the m7G-capped substrate, methylation at cap 0 results in a mobility shift after exposure to rTbMTr1, indicating that cap 0 methylation is not necessary for rTbMTr1 2'-O-ribose methylation activity.

To determine whether rTbMTr1 acts preferentially on methylated or unmethylated guanosine-capped substrates, time course kinetics were performed over 60 min using m7G*KpppApGp-SL and G*KpppApGp-SL. rTbMTr1 showed similar affinity for the methylated or unmethylated G-capped RNA substrates (Fig. 3C). Either 300 fmol of m7G*KpppApGp-SL or 270 fmol of G*KpppApGp-SL substrate was methylated by 2 pmol of rTbMTr1 in 30 min with an estimated turnover of

FIGURE 1. Purification and activity of recombinant TbMTr1. SDS-PAGE analysis of protein profiles during purification of rTbMTr1 is shown in supplemental Fig. S1. A, cap 1 2'-O-ribose MTase activity assay. 32P-Labeled methyl N7-G-capped substrate was incubated with (+) or without (−) the addition of purified rTbMTr1. RNA samples extracted from the reactions were treated with nuclease P1 to release the cap structures and their methylation status was analyzed by TLC on a polyethyleneimine-cellulose sheet. Positions of methylated and unmethylated cap markers are indicated on the right. B, products from reactions with (+) or without (−) the co-factor AdoMet, using m7G*KpppApGp-SL RNA as substrate were digested with RNase T2 and electrophoresed through a 25% urea-polyacrylamide gel containing 8 M urea.
0.005 min⁻¹. 

Properties of cap 1 ribose methylation

Although rTbMTr1 can act upon both methylated and unmethylated G-capped RNA substrates in vitro, the m⁷G-cap 0 RNA is likely to be its primary substrate. The recent identification of the bifunctional TbCgm1 containing both guanylation and guanosine N² methyltransferase activity and its role in SL RNA cap formation indicates that the unmethylated G-capped substrate is a short-lived intermediate. Whether the U1 snRNA is also capped by TbCet1/TbCgm1 or the three-component TbCet1/TbCgm1/TbCmt1 is unclear, but rTbMTr1 recognizes both RNA substrates.

TbMTr1 Is Related to Metazoan MTases—TbMTr1 is from a large family of Rossmann-fold MTases (18, 20, 32). PSI-BLAST searches performed with the TbMTr1 amino acid sequence using a stringent expectation (e) value of 10⁻²⁰ as cut-off yielded about 20 unique protein sequence hits mainly from baculoviral and higher eukaryotic origins, including Drosophila, C. elegans, and human. All the hits are predicted to contain RrmJ-like MTase domains. RrmJ protein is a 2'-O-ribose MTase that modifies 23S rRNA in E.coli (33). Protein sequence alignments done with MUSCLE (29) for the 370-amino acid (aa) T. brucei TbMTr1 protein (Tb) and its homologs from fellow kinetoplastids T. cruzi (Tc; 362 aa), GeneDB Tc00.1047053506247.320 and L. major (Lm; 400 aa, LmjF36.6660) showed sequence conservation (Fig. 4). The homologs from Drosophila (788 aa), C. elegans (927 aa), and human (882 aa) were larger and shared a number of conserved regions in the predicted RrmJ-like domain. Similar observations were made for the AcNPV orf69 protein and the Spodoptera litura nucleo-polypolyhedrovirus TbMTr1 homologs. The conserved regions correspond to the nine motifs typical of AdoMet-dependent MTases (34).

Amino acids in the motif I region are involved in binding the methionine moiety of AdoMet that acts as a methyl group donor in the MTase reaction (35–37). The conservation in the motif I region with five invariant amino acids supported an AdoMet binding domain in these

**TABLE 1**

Properties of cap 1 2'-O-methyltransferases

| Eukaryotes and baculovirus | Poxxvirus, AcNPV orf69 | Flaviviruses | Mononegavirales |
|-----------------------------|------------------------|--------------|-----------------|
| T. brucei TbMTr1            | Human MTase 1          | Vaccinia VP39 | Dengue NS5      |
| 2'-O-MTase                  | +                      | +            | +               |
| N7-MTase                    | -                      | -            | -               |
| 5' cap preference           | G & m⁷G                | G & m⁷G      | G & m⁷G         |
| N1 preferred               | A > G                   | A = G = C    | A               |
| N2 preferred               | A = G                   | ND           | ND              |
| pH optimum                  | ≥ 9                    | ND           | ND              |
| Localization               | Nucleus                | Nucleus      | Cytosol         |
| Crystal structure           | No                     | No           | Yes             |
| References                  | This study             | 22           | Yes             |

a ND, not determined.
b N1 preferred, base preference at position 1.
c Only G tested.
d N2 preferred, base preference at position 2.
Sequence-specific mRNA Cap 1 Ribose Methylation

A - + - + TbMTr1 B - + TbMTr1
<\(\text{m}^7\text{G}\)*pppA<br>
<\(\text{m}^7\text{G}\)*p<br>
<\(\text{G}\)*ppAm<br>
<\(\text{G}\)*pppA

G cap m\(^7\)G cap

C

![Graph showing methylation activity vs. time](image)

\[\text{m}^7\text{G}\text{pppA} \text{or GpppAm (molecules)} \times 10^4 \text{against time.} \]

\[\text{Time (min)} \times 60 \leq 3600 \text{ against mL units.} \]

FIGURE 3. \(r\text{TbMTr1}\) methylates both \(G\)-capped and \(\text{m}^7G\)-capped RNA substrates. Standard \(r\text{TbMTr1}\) assays were carried out using \(G\text{pppApGp-SL}\) or \(\text{m}^7G\text{pppApGp-SL}\) RNA as substrates with or without the addition of \(r\text{TbMTr1}\). A, substrate \(N\)-\(G\) cap methylation. Reaction products were digested with 0.2 units of TAP and released cap structures were separated by TLC on polyethylenimine-cellulose sheets developed with 0.45 M ammonium sulfate. Migration positions of markers are marked on the right. B, cap 1 2\(^{\prime}\)-O-ribose MTase activity assay. \(\text{\[^32P\]-Labeled unmethylated G-capped RNA (G cap) substrate was incubated with (+) or without (−) the addition of purified rTbMTr1 in the standard reaction mixture. Radiolabeled dinucleotide cap structures released by P1 nuclease were resolved by TLC chromatography. Positions of methylated and unmethylated cap markers are indicated on the right. C, time course kinetics comparing the activity of \(r\text{TbMTr1}\) on \(G\text{pppApGp-SL}\) (○) and \(\text{m}^7G\text{pppApGp-SL}\) (●) RNA substrates. \(r\text{TbMTr1}\) assay reactions were scaled up 10 times to a 200-μl volume, and 20-μl samples were taken out at the indicated times. Extracted RNA was treated with nuclease P1 to release the cap structures and their methylation status was analyzed by TLC on a polyethylenimine-cellulose sheet. The extent of cap methylation is plotted against time.

proteins. The alignment also showed the conservation of two lysine residues in motif X and motif VI, an aspartic acid residue in motif IV, and a glutamic acid residue in motif VIII (Fig. 4, white letters on black) that corresponded to the characteristic KDKE catalytic tetrad found in nearly all 2\(^{\prime}\)-O-ribose MTases from the RrmJ family (20, 38).

Based on our bioinformatics analyses and because AcNPV orf 69 encodes a functionally characterized cap 1 2\(^{\prime}\)-O-ribose MTase (22), we propose a similar structure for TbMTr1 and its homologs. The function of TbMTr1 has been challenged genetically by our \textit{in vivo} studies (18), and the catalytic center will be examined here \textit{in vitro}. The structural locations identified by this bioinformatic analysis were used to target specific positions within the protein for mutagenesis.

\textit{K-D-K-E Mutations Abrogate TbMTr1 Activity}—Amino acid residues Lys\(^95\), Asp\(^207\), Lys\(^248\), and Glu\(^285\) (Lys\(^95\), Asp\(^207\), Lys\(^248\), Glu\(^285\)) were identified as the potential catalytic tetrad in the active site based on our sequence alignments. To challenge the validity of the prediction, we expressed and purified mutated rTbMTr1 proteins containing alanine substitutions for Lys\(^95\), Asp\(^207\), Lys\(^248\), or Glu\(^285\) (supplemental Fig. S3). Considering the importance of the K-D-K-E tetrad for functionality in known 2\(^{\prime}\)-O-ribose MTases like VP39, we anticipated that mutation of these individual residues would inhibit rTbMTr1 activity. Analysis of the purified mutated proteins using \(\text{m}^7G\text{pppApGp-SL}\) RNA as substrate showed that all proteins were severely reduced in the ability to methylate the substrate RNA (Fig. 5). The K95A and K248A mutated proteins resulted in >98% inhibition of activity, whereas D207A and E285A mutated proteins were inhibited by 96 and 93%.

Our prediction for the K-D-K-E tetrad in the active domain of TbMTr1 is validated experimentally, implying that these residues will fall in close proximity to one another in the folded protein. The results agree qualitatively with the mutational analysis of RrmJ, in which the glutamic acid residue was found to be the least important in the K-D-K-E tetrad (39).

\(r\text{TbMTr1}\) Binds to Unmethylated and Monomethylated but Not TMG Cap 0—Both SL and U1 snRNAs are substrates for \(r\text{TbMTr1}\) \textit{in vitro} (18). Whereas the SL RNA has a \(\text{m}^7G\)-cap, U1 snRNA is hypermethylated (\(\text{m}^{2,2,7}G\)). Limited activity is seen on dinucleotide cap analogs for some cap 1 MTases, e.g., VP39 (40). Dinucleotide cap analogs were tested as substrates, with high concentrations used to allow for the detection of low level activity. To maximize the efficiency of the reaction, each dinucleotide was present at 1 mM concentration with 47 pmol of \(r\text{TbMTr1}\) and 55 mM \[^3H\]AdoMet (Amersham Biosciences). No 2\(^{\prime}\)-O-ribose methylation activity was detected (data not shown); this result indicated that a longer substrate RNA chain was required for activity. The inability of the dinucleotide analogs to serve as methylation substrates led to their use as cap binding competitors in the methylation of our standard 150-nucleotide substrates.

To understand the requirements for cap binding and position 1 identity for \(r\text{TbMTr1}\) activity, increasing concentrations of various unlabeled dinucleotide cap analogs were included in the \(r\text{TbMTr1}\) reaction mixture as competitors (Fig. 6A). Monomethylated cap analogs \(\text{m}^7G\text{pppG}\) and \(\text{m}^7G\text{pppA}\) inhibited MTase activity by about 40% at 0.1 mM concentration, and 66 and 85%, respectively, at 1 mM concentrations. The unmethylated cap analog \(\text{GpppG}\) was less effective, causing 24 and 42% inhibition at 0.1 and 1 mM concentrations. The bidirectional cap analog \(\text{m}^{2,2,7}G\text{pppA}\) was most effective, yielding 75 and 95% inhibition at 0.1 and 1 mM concentrations. No significant reduction in activity was observed with GTP or the TMG cap analog \(\text{m}^{2,2,7}G\text{pppA}\) even at 1 mM concentrations, demonstrating that the TMG cap is not bound by \(r\text{TbMTr1}\). The high molar requirements for the cap analogs \(\text{m}^7G\text{pppA},\)}
m'GpppG, or GpppG to inhibit rTbMTr1 indicated a low binding affinity for dinucleotide substrates. The inhibitory effects of m'GpppA and m'GpppG were resolved between 0.05 and 1 mM to visualize the fine kinetics of each competitor (Fig. 6, B and C).

About 90% inhibition of rTbMTr1 activity was observed with 0.25 mM m'GpppA, whereas a 4-fold higher concentration of m'GpppG was required for a similar level of inhibition.

Adenosine is the first transcribed nucleotide for both SL and U1 snRNAs in trypanosomes, and the higher efficacy of m'GpppA over m'GpppG as inhibitor indicates a preference for adenine. The m'G-capped species have a greater competitive edge, whereas the lack of inhibition by the TMG analog implies that an intermediate G- or m'G-capped version of U1 snRNA is the substrate for TbMTr1.

rTbMTr1 Prefers A over G at Position 1—Additional nucleotides from the substrate RNA were required for enzyme activity, similar to the requirements of the WNV NS5 2'-O-ribose MTase that methylates viral RNA specifically. Thus we examined the primary sequence of the substrate for modulation of rTbMTr1 activity. The SL-based substrate was modified only at position 1 or as a whole, with all transcripts maintaining the guanosine at position 2 to foster efficient in vitro transcription.

Time course experiments performed over 60 min to monitor cap 1 methylations by nuclease P1 digestion showed a time-dependent conversion of m'G*pppA to m'G*pppAm for the m'G*pppApGp-SL RNA, with 60% of the cap structures migrating faster than the mGpppA marker (Fig. 7A). A non-SL RNA substrate m'G*pppGpGp-N was synthesized using a PCR-amplified DNA fragment corresponding to nucleotides 622 to 793 of plasmid pBluescript SK(+) (Stratagene) as template. The m'G*pppGpGp-N RNA begins with mGpppGpGpGpCpUp and has a chain length of 150 nucleotides. No significant change in mobility for the nuclease P1-liberated cap structures was seen over time for the m'G*pppGpGp-N RNA, indicating a substrate specific activity for rTbMTr1.

To determine whether the identity of the methyl-acceptor nucleotide at the N1 position is crucial for activity (Fig. 7B), a synthetic RNA m'G*pppGpGp-SL with an A1 to G1 change was used as substrate. The 2'-O-ribose methylation activity was reduced to 11% the level of the m'G*pppApGp-SL substrate, suggesting a methyl acceptor nucleotide preference for A. To extend this observation, we synthesized the pBluescript sequence-specific transcript m'G*pppApGp-N, with a single
nucleotide substitution of G1 to A1. Modification of A1 in this otherwise non-SL substrate was evident from the TLC chromatogram, with a 23% rate of conversion of \( \text{m}^7\text{G}^*\text{pppAm} \) relative to the \( \text{m}^7\text{G}^*\text{pppApGp-SL} \) substrate at 60 min. The four activity curves are plotted for comparison in Fig. 7C. Other possible nucleotides as position 1 methyl acceptors were not tested due to the lack of a suitable \textit{in vitro} transcriptional system that can initiate with pyrimidines. Adenine plays a major role in determining the rate of rTbMTr1 activity, as is evident from a 9-fold reduction in position 1-specific methylation due to an A1 to G1 change in the SL substrate, as well as a 4-fold enhancement of the activity with a G1 to A1 change in the plasmid substrate. Downstream nucleotides are implicated in substrate-enzyme interaction as the A1 to G1 change in SL sequence was not sufficient to abolish the TbMTr1 activity, and the G1 to A1 change in the plasmid substrate sequence did not restore full activity. With the identity of position 1 fixed at A, we next sought to challenge downstream positions for variance from the SL RNA.

**SL Sequence Maximizes rTbMTr1 Activity**—Past \textit{in vivo} studies mutagenizing the first four transcribed nucleotides pppAACU of the SL RNA have implicated position 3 in the cap 4 formation (13). To assess if the cap 1 activity in particular is sensitive to the identity of these nucleotides, we examined position 2 and 3 modifications specifically.

The second nucleotide of the \( \text{m}^7\text{GpppAgCU-SL} \) substrate was substituted with each of the other three nucleotides,
including the naturally occurring SL RNA A₂ that was replaced by G for enhanced in vitro transcriptional efficiency. The m⁷GpppAgCU-SL substrate was methylated almost as efficiently as the wild-type m⁷GpppAACU-SL substrate, whereas A₂ to U₂ and A₂ to C₂ mutations resulted in 50 and 40% reduction in the extent of 2'-O-methylation. Mutating C₃ to G₃ or U₃ resulted in 25 and 35% inhibition, respectively, whereas the A₃ substrate was methylated as efficiently as wild-type C₃.

Enzyme-substrate interaction domains in rTbMTr1 recognize nucleotides at the 5' end of the substrate beyond position 1. Purines are favored at position 2. At position 3 the wild-type SL C can be replaced efficiently by an adenine, but G or U substitutions show reduced suitability for the enzyme. The TbMTr1 amino acids involved in these interactions have yet to be identified.

**DISCUSSION**

The 2'-O-ribose MTases, TbMTr1, TbMTr2, and TbMTr3, involved in the SL cap 4 formation in *T. brucei* (Fig. 9) share a conserved catalytic domain diagnostic of AdoMet-dependent MTases. Phylogenetic analysis of the three MTases shows distinct evolutionary pathways with TbMTr2 and TbMTr3 related to the poxvirus cap 1 MTase VP39, and TbMTr1 related to predicted 2'-O-ribose MTases from higher eukaryotes and double-stranded viruses (20). TbMTr2 and TbMTr3 generate cap 2 and cap 3 modifications, respectively. As such, their activity is distinct from that of VP39, which generates the cap 1 modification on vaccinia virus mRNA. We have shown genetically that TbMTr1 is responsible for 2'-O-ribose methylation of the first transcribed nucleotide of both SL and U1 snRNA, and that rTbMTr1 can transfer a methyl group to a synthetic SL RNA (18). We report here the detailed biochemical characterization of rTbMTr1. The cap 1 2'-O-MTases have a range of differing enzymatic and substrate-recognition characteristics, and a summary of their properties is presented in Table 1.

The cap 1 2'-O-MTases differ in their ability to use methylated and unmethylated guanosine-capped RNAs as substrates. Both G-capped (GpppAACU-SL) and m⁷G-capped (m⁷GpppAACU-SL) RNA substrates were methylated by rTbMTr1 with comparable kinetics, showing that activity minimally requires a guanosine cap on its RNA substrate, and is independent of the guanosine cap N⁷-methylation. With the exception of vaccinia virus VP39 protein, all other viral cap 1 MTases catalyze ribose methylation at position 1 independent of the N⁷-guanosine cap methylation status. VP39 binds to both methylated and unmethylated guanosine-capped substrate, but specifically requires a N⁷-methylated guanosine cap for activity (19). Although rTbMTr1 shows comparable affinities for both unmethylated and N⁷-methylated guanosine-capped substrates (estimated *Kₘ* values 33.3 and 38 nM, respectively), increased inhibition by the m⁷G dinucleotide analogs suggests differential recognition of the cap 0 forms. Binding to the guanosine cap of substrate RNA alone is not sufficient for rTbMTr1 activity. The dinucleotide cap analogs inhibited rTbMTr1 activity when included as competitors in the assay mixture at high concentrations, but were not viable substrates. In addition to the guanosine cap, the length and nucleotide composition of substrate RNA may play a role in enzyme-substrate complex formation. The human cap 1 MTase activity from HeLa cell extracts requires minimally a trinucleotide substrate.
Sequence-specific mRNA Cap 1 Ribose Methylation

A

\[ \text{A} \]

\[ \text{ACU-SL, ACU-SL, ACU-SL, ACU-SL, ACU-SL} \]

\[ \text{AgCU-SL, AgCU-SL, AgCU-SL, AgCU-SL, AgCU-SL} \]

\[ \text{< m^G*pppAm} \]

\[ \text{< m^G*pppA} \]

for the transfer of a methyl group \((3)\), whereas the \(K_m\) for VP39 increased with substrate length \((41)\). The mechanism of cap recognition by TbMTr1 is being addressed currently by mutational and structural studies.

A second feature of the cap 1 2'-O-ribosyltransferase activity of rTbMTr1 contrasts the properties of the T. brucei cap 2-specific 2'-O-ribosyltransferase activity of rTbMTr1 (Ref. 18 and this study); cap 2 modification by TbMTr2/TbCom1/TbMT48 (14, 16, 17); and cap 3 modification by TbMTr3/TbMT37 (14, 15). The enzyme responsible for cap 4 ribose methylation has not been identified, however, TbMTr2/TbMT27 is a candidate (dotted line). The enzymes responsible for base methylations (open circles) have not been identified.

FIGURE 8. Substrate position effects at nucleotides 2 and 3. A, effect of nucleotide sequence at the 5' end of substrate RNA was tested with cap-labeled SL RNA substrates containing changes in the second or third nucleotides (indicated in lowercase) in standard rTbMTr1 reactions. B, quantitation of the position-specific effects is shown in histogram form.

The high pH optimum is a characteristic of many cap 1 2'-O-ribose MTases. Structural and mutagenesis studies of VP39 and RrmJ 2'-O-ribose MTases show that the KDKE tetrad in the catalytic site of the enzyme mediates a \(S_o\)-type transfer of the methyl group that involves the deprotonation of the target 2'-OH group. The deprotonated 2'-OH group then attacks the methyl moiety of the donor AdoMet to accomplish the methyl transfer \((39, 43)\). A prediction for the signature KDKE tetrad in rTbMTr1, critical for functioning of all AdoMet dependent 2'-O-ribose MTases, was experimentally challenged by site-specific mutation. Individual alanine substitutions of the functional KDKE tetrad proved to be lethal for rTbMTr1 activity, validating their assignments and suggesting a similar mode of activity. However, VP39 and its trypanosome ortholog TbMTr2 differed in substrate and pH requirements. The optimum pH for both VP39 and TbMTr2 (TbCom1) are around 7.5. Although a defined optimal pH range could not be determined for TbMTr1, the activity was found to increase continually up to the highest tested pH 9.5. A 4-fold increase in turnover was observed between reaction mixtures at pH 8.0 \((0.003 \text{ min}^{-1})\) and 9.5 \((0.013 \text{ min}^{-1})\). High pH requirement for optimal activity was also reported for the HeLa cap 1 MTase as well as for the 2'-OMTase activity of the WNV NS5 protein in \textit{in vitro} assay systems. Interestingly, the N\(^7\)-guanylyltransferase
activity of the WNV NS5 protein is optimal at pH 7.5 suggesting that the two functions are mechanistically different. Based on the structural analysis of WNV NS5 and VP39 proteins, the high pH has been proposed to facilitate the S_{2}′-like transition state during the methyl transfer reaction through effective deprotonation of lysine 182 in the catalytic tetrad (31). The significance of high pH requirement for rTbMTr1 activity is not clear at present due to the lack of detailed structural information for this enzyme or its close relatives. Although other effects of high pH on the structure of the active core cannot be ruled out, the lysine model is consistent with our mutagenesis data.

Several common biochemical features support the link between the HeLa cell cap 1 MTase and rTbMTr1. Both enzymes show a higher pH requirement for optimal activity. The rTbMTr1 and the human cap 1 MTase show similar cap 0 substrate specificities, although the human enzyme showed no position 1 nucleotide preference as it could methylate an adenosine or a cytosine at position +1 with equal efficiency (3). Both enzymes failed to methylate capped dinucleotide substrates, but the minimal substrate size for rTbMTr1 has yet to be determined. A TbMTr1-GFP fusion protein localizes to the nucleus (18); whereas the HeLa cap 1 MTase was detected in both cytosolic and nuclear fractions, the specific activity in the nuclear fraction was 8-fold higher (3), as would be anticipated for a nuclear enzyme.

In the emerging model for SL cap biogenesis, individual methylations leading to cap 4 formation may occur independently (14–18), as opposed to being a rigid cascade of events. The spatial partitioning of the SL maturation pathway may impose a methyl transfer reaction through effective deprotonation of lysine 182 in the catalytic tetrad (31). The significance of high pH requirement for rTbMTr1 activity is not clear at present due to the lack of detailed structural information for this enzyme or its close relatives. Although other effects of high pH on the structure of the active core cannot be ruled out, the lysine model is consistent with our mutagenesis data.

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Sequence-specific mRNA Cap 1 Ribose Methylation

U1 snRNA from further modifications in the cap 4 pathway. The enzyme(s) responsible for the position 1 base dimethylation of the SL and/or U1 snRNA has not been identified.

**TbMTr1** is not an essential gene for *T. brucei* cell survival, with no apparent change in the growth phenotype of knock-out organisms (18). In the nematode *Ascaris lumbricoides* cap 1 is not required for trans-splicing (46); however, it is involved in translational efficiency (47). Knockdown of a TbMTr1 homologue in *C. elegans* causes maternal sterility (48), but the normal cap status of the SLs in this system have yet to be determined. Both of the nematodes use TMG-capped SLs in their trans-splicing pathways, transcribed from RNA pol II promoters and beginning with ppGpGpG. If the nematode MTr1 retains similar cap-binding constraints to those seen in the kinetoplastids, their cap 1 2′-O-ribose methylation may occur on mG-capped intermediate SL substrates prior to TMG-cap formation, and some level of substrate preference, in this case for the G nucleotide, may also be found. Further investigation of the *TbMTr1* family will facilitate understanding of the functional role of mRNA cap structures in kinetoplastids and in higher eukaryotic systems.

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