

# Functional characterization of a 48 kDa *Trypanosoma brucei* cap 2 RNA methyltransferase

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## ABSTRACT

Kinetoplastid mRNAs possess a unique hypermethylated cap 4 structure derived from the standard m<sup>7</sup>GpppN cap structure, with 2'-O methylations on the first four ribose sugars and additional base methylations on the first adenine and the fourth uracil. While the enzymes responsible for m<sup>7</sup>GpppN cap 0 formations has been characterized in *Trypanosoma brucei*, the mechanism of cap 4 methylation and the role of the hypermethylated structure remain unclear. Here, we describe the characterization of a 48 kDa *T.brucei* 2'-O nucleoside methyltransferase (TbCom1). Recombinant TbCom1 transfers the methyl group from S-adenosylmethionine (AdoMet) to the 2'-OH of the second nucleoside of m<sup>7</sup>GpppNpNp-RNA to form m<sup>7</sup>GpppNpNmp-RNA. TbCom1 is also capable of converting cap 1 RNA to cap 2 RNA. The methyl transfer reaction is dependent on the m<sup>7</sup>GpppN cap, as the enzyme does not form a stable interaction with GpppN-terminated RNA. Mutational analysis establishes that the TbCom1 and vaccinia virus VP39 methyltransferases share mechanistic similarities in AdoMet- and cap-recognition. Two aromatic residues, Tyr18 and Tyr187, may participate in base-stacking interactions with the guanine ring of the cap, as the removal of each of these aromatic side-chains abolishes cap-specific RNA-binding.

## INTRODUCTION

The 5' cap of eukaryotic mRNAs is essential for protecting mRNA from degradation and facilitating translation initiation. The simplest cap structure, m<sup>7</sup>GpppN or cap 0, consists of an N-7 methylated guanosine linked by a 5'-5' triphosphate bridge to the first nucleoside of nascent transcripts. In higher eukaryotes, including mRNAs from insects, vertebrates and their viruses, cap 0 is further modified by the addition of

methyl groups to either the ribose or base moiety of the first and the second transcribed nucleosides [reviewed in (1)]. Methylation at the 2'-hydroxyl position of the first transcribed ribose is termed cap 1 (m<sup>7</sup>GpppNm) and similar methylation of the second transcribed position is termed cap 2 (m<sup>7</sup>GpppNmNm). While cap-specific 2'-O nucleoside RNA methyltransferases have been identified from viral sources and the activity has been detected in mammalian extracts (2–5), the significance of the methylated cap structure and the role of these methyltransferases in cellular mRNA metabolism remain largely unexplored.

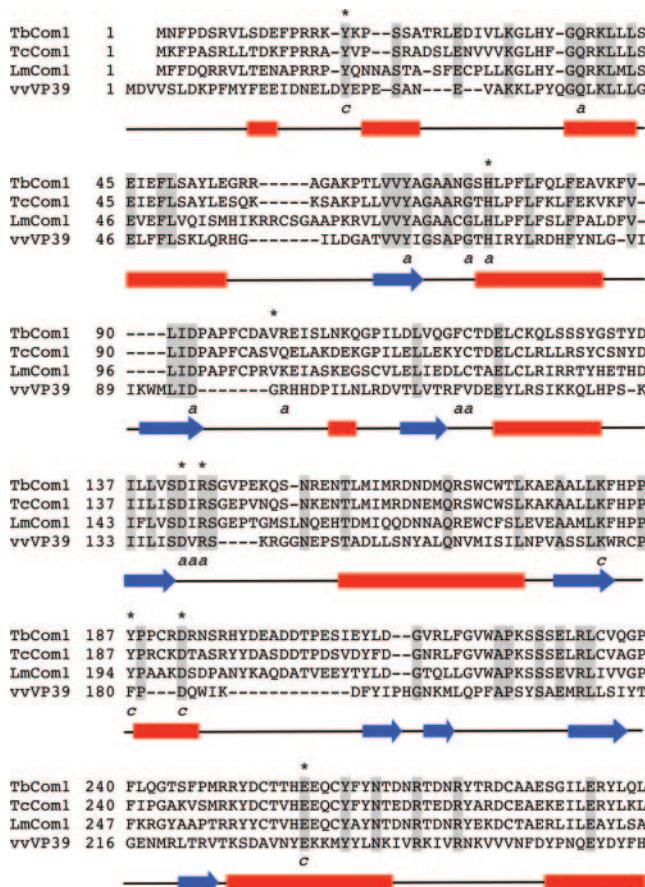
*Trypanosoma brucei* and other related kinetoplastids possess a unique hypermethylated cap structure called cap 4, which consists of a standard cap 0 with 2'-O methylations on the first four ribose sugars (A<sub>m</sub>A<sub>m</sub>C<sub>m</sub>U<sub>m</sub>), and additional base methylations on the first adenine (m<sup>6,6</sup>A) and the fourth uracil (m<sup>3</sup>U) (6,7). The cap 4 structure is formed exclusively on the spliced leader (SL) RNA and is transferred to individual pre-mRNAs derived from a polycistronic transcript by trans-splicing to form mature mRNAs (8–10). Analysis of SL RNA biogenesis suggests that cap 4 is derived from cap 0 and is formed cotranscriptionally by sequential methylation in a 5'–3' direction, and that the methylation steps that lead to cap 4 are required for the trans-splicing reaction (7,11,12). Recently, a *T.brucei* nuclear cap-binding protein (TbCbc), which facilitates mRNA maturation and translation, was found to bind to cap 4 RNA with higher affinity than cap 0 RNA (13). These findings suggest that cap 4 plays an important role in mRNA biogenesis in trypanosomes, although it does not appear to be required for SL RNA stability, ribonucleoprotein particle assembly, or formation of SL RNA secondary structure *in vivo* (12).

A comprehensive genomic sequence analysis of 2'-O RNA methyltransferase family members identified two open reading frames in the *T.brucei* genome, Tb11.02.2500 (48 kDa) and Tb09.211.3130 (57 kDa), that resemble the vaccinia virus VP39 protein (14). VP39 is a prototype of cap-dependent 2'-O nucleoside methyltransferase; it transfers a methyl group from AdoMet to the ribose 2'-OH of the first transcribed nucleoside of viral mRNA to form cap 1 (15). Over twenty X-ray crystal structures of the VP39 protein have been solved complexed with AdoMet, S-adenosylhomocysteine (AdoHcy), cap analogues and m<sup>7</sup>Gppp(A)<sub>5</sub> oligomers, as well as various methylated nucleotides

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(16–20). Residues that contact AdoMet and the  $m^7G$  moiety in the VP39 co-crystal structures are conserved in the 48 and 57 kDa *T. brucei* proteins, suggesting that these *T. brucei* proteins may be responsible for SL RNA cap 4 formation.

In this study, we present biochemical characterization of the 48 kDa *T. brucei* protein's cap-dependent 2'-O methyltransferase activity. We named this protein *TbCom1* (*T. brucei* cap 2'-O methyltransferase) to denote the enzyme's function. *TbCom1* homologs are encoded in *Trypanosoma cruzi* (*TcCom1*) and *Leishmania major* (*LmCom1*) (Figure 1). Recombinant *TbCom1* binds specifically to  $m^7GpppN$ -terminated RNA and catalyzes the transfer of a methyl group from AdoMet to the 2nd transcribed nucleoside to form  $m^7GpppNpNm$ . Mechanistic conservation between VP39 and *TbCom1* in AdoMet and cap 0 recognition is suggested by structure-based mutational analysis of putative AdoMet- and cap-binding residues.



**Figure 1.** Sequence conservation among vaccinia and kinetoplast cap-dependent 2'-O nucleoside methyltransferases. The amino acid sequences of *TbCom1* and *T. cruzi* (*TcCom1*: XP\_81711), and *L. major* (*LmCom1*: CAJ03958) homologs are aligned to the sequence of vaccinia virus VP39. Side-chain identity in all proteins is highlighted by shaded boxes. Gaps in the alignment are indicated by dashes. VP39 amino acid side-chains that contact AdoMet or the  $m^7G$  cap are indicated by 'a' and 'c', respectively. The secondary structure of VP39 is indicated below the aligned sequences. The  $\beta$ -strands are depicted as arrows and  $\alpha$ -helices as bars. *TbCom1* residues that were mutated to alanine in this study are denoted by asterisks above the sequence.

## MATERIALS AND METHODS

### Cloning, expression and purification of recombinant *TbCom1*

The *Tb11.02.2500* (*TbCOM1*) open reading frame was amplified by PCR from total *Trypanosoma brucei brucei* genomic DNA (a gift from Laurie Read, SUNY Buffalo) using oligonucleotide primers designed to introduce an *NdeI* restriction site at the predicted translation start codon and a *BglII* site 3' of the predicted stop codon. The 1.3 kb PCR product was digested with *NdeI* and *BglII* and inserted into pET16b (Novagen) to generate the plasmid pET-*TbCOM1*. In this context, *TbCOM1* is fused in frame with a 20 amino acid N-terminal leader peptide containing 10 tandem histidines and expression of the His-tagged protein is driven by the T7 RNA polymerase promoter. The predicted amino acid sequence of the 417 amino acid *TbCom1* protein is shown in Figure 1.

Alanine-substitution mutations were introduced into the *TbCOM1* gene by PCR using the two-stage overlap extension method. The residues targeted for alanine-mutagenesis include Tyr18, His74, Val115, Asp142, Arg144, Tyr187, Asp192 and Glu253. The pET-*TbCOM1* plasmid was used as the template for the first-stage amplifications. The DNA products of the second-stage amplification were digested with *NdeI* and *BglII* and inserted into pET16b. The presence of the desired mutation was confirmed by DNA sequencing analysis, and the presence of unwanted mutations was excluded by sequencing the entire gene.

Wild-type and mutant pET-*TbCOM1* were transformed into *Escherichia coli* BL21(DE3) Gold (Novagen). A 2 l culture of *E. coli* BL21(DE3) Gold/pET-*TbCOM1* was grown at 37°C in Luria-Bertani medium (LB medium) containing 0.1 mg/ml ampicillin until the OD<sub>600</sub> reached 0.4. Isopropyl-D-thiogalactopyranoside (IPTG) was added to 0.4 mM and growth was continued at 17°C for 18 h. Cells were harvested by centrifugation and stored at -80°C. All subsequent procedures were performed at 4°C. Thawed bacteria were resuspended in 100 ml of Buffer A [50 mM Tris-HCl (pH 7.5), 10% sucrose and 0.25 M NaCl] and lysed by the addition of lysozyme and Triton X-100 to final concentrations of 100  $\mu$ g/ml and 0.1%, respectively. The lysate was sonicated to reduce viscosity, and insoluble material was removed by centrifugation for 45 min at 14000 r.p.m. in a Beckman TA14-50 rotor. The soluble extract was applied to a 5 ml column of Ni-NTA-agarose resin (Qiagen) that had been pre-equilibrated with Buffer A containing 0.1% Triton X-100. The column was washed with 50 ml of the same buffer and eluted stepwise with 15 ml aliquots of Buffer B [50 mM Tris-HCl (pH 8.0), 0.25 M NaCl and 10% glycerol] containing 0.05, 0.1, 0.2, 0.5 or 1 M imidazole. The polypeptide composition of the column fractions was monitored by SDS-PAGE. The His-tagged *TbCom1* proteins adsorbed to the column and were recovered in 0.1, 0.2 and 0.5 M imidazole eluates. These fractions were pooled and adjusted to 0.025 M NaCl by addition of Buffer C [50 mM Tris-HCl (pH 8.0) and 10% glycerol], and then applied to a 5 ml column of diethylaminoethyl (DEAE) cellulose pre-equilibrated in Buffer C containing 0.025 M NaCl. *TbCom1* did not bind to DEAE at low ionic strength but many of the bacterial polypeptides were eliminated during this step. The DEAE

flowthrough fraction was applied to a 5 ml phosphocellulose column pre-equilibrated in Buffer B with 0.025 M NaCl. The column was washed with 50 ml of the same buffer and then eluted stepwise with 15 ml of Buffer B containing 0.05, 0.1, 0.2, 0.3, 0.5 and 1.0 M NaCl. TbCom1 was recovered predominantly in the 0.2 M NaCl fraction (~5 mg). The protein sample was concentrated using Amicon-Ultra centrifugal filter devices (Millipore) to 2.4 mg/ml, stored at  $-80^{\circ}\text{C}$  and thawed on ice just prior to use. Protein concentrations were determined with the Bio-Rad dye reagent using BSA as the standard.

### Expression and purification of the vaccinia virus VP39 protein

The VP39 expression plasmid, pET42a-J3R, was a generous gift from Ed Niles (SUNY Buffalo). pET42a-J3R was introduced into *E. coli* BL21(DE3) Gold and grown at  $37^{\circ}\text{C}$  in LB medium containing 0.1 mg/ml ampicillin until the  $\text{OD}_{600}$  reached 0.4. The culture was adjusted to 0.4 mM IPTG and incubation was continued for 4 h. VP39 was purified from soluble bacterial lysate by one-step nickel-agarose chromatography. The 0.2 M imidazole eluate fractions containing VP39 (2.8 mg/ml) were stored at  $-80^{\circ}\text{C}$  and thawed on ice just prior to use.

### Preparation of RNA substrates

Triphosphate-terminated [ $\alpha$ - $^{32}\text{P}$ ]GTP-labeled RNAs were synthesized by *in vitro* transcription from PCR amplified linear DNA templates containing the T7 RNA polymerase promoter in the presence of 2 mM [ $\alpha$ - $^{32}\text{P}$ ]GTP, ATP, CTP and UTP. RNAs were purified on a non-denaturing 8% polyacrylamide gel and eluted overnight in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, then precipitated with ethanol. The molar concentration of  $^{32}\text{P}$ -labeled RNA was calculated according to the specific activity of the input [ $\alpha$ - $^{32}\text{P}$ ]GTP. Fifty picomoles of *in vitro* synthesized RNA were capped in a reaction mixture (100  $\mu\text{l}$ ) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 2.5 mM  $\text{MgCl}_2$ , 50  $\mu\text{M}$  AdoMet (Sigma), 50  $\mu\text{M}$  GTP, 100 pmol of mammalian capping enzyme (Mce1) and 100 pmol of recombinant yeast guanine N-7 methyltransferase (Abd1) at  $30^{\circ}\text{C}$  for 1 h (21,22). Proteins were removed by phenol-chloroform (1:1) extraction and the  $\text{m}^7\text{GpppRNAs}$  were recovered by ethanol precipitation. For preparation of cap 1-terminated RNA ( $\text{m}^7\text{GpppGmp-RNA}$ ), 100 pmol of recombinant vaccinia VP39 was added to the above reaction. Unmethylated capped RNA (GpppRNA) was prepared by omitting Abd1 and AdoMet from the above reaction.

### Cap methyltransferase assay

Standard reaction mixtures (10  $\mu\text{l}$ ) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 1 pmol of either  $^{32}\text{P}$ -labeled  $\text{m}^7\text{GpppRNA}$  (cap 0) or  $\text{m}^7\text{GpppGmRNA}$  (cap 1) and 4 pmol of TbCom1 were incubated for 30 min at  $27^{\circ}\text{C}$ . The reaction mixtures were adjusted to 50  $\mu\text{l}$  by addition of 50 mM Tris-HCl (pH 7.5) and 10  $\mu\text{g}$  glycogen, and the RNA was extracted by phenol-chloroform (1:1) followed by ethanol precipitation. The pellet was resuspended in 10  $\mu\text{l}$  of water and treated with either 140 ng nuclease P1 (US Biologicals) in 50 mM sodium acetate (pH 5.5) or RNase cocktail (330 ng of RNase A, 0.33 U of RNase T1 and

330 U of RNase T2) (Sigma) in 50 mM ammonium acetate and 1 mM EDTA at  $37^{\circ}\text{C}$  for 16 h. While both nuclease P1 and RNase hydrolyze the phosphodiester bonds in RNA, nuclease P1 generates 5' phosphate mononucleotides whereas RNase generates 3' phosphate mononucleotides. Two microliters of the above reactions were mixed with an equal volume of 90% formamide and 10 mM EDTA and the products were resolved on denaturing 22% polyacrylamide gels containing 7 M urea in 0.5 $\times$  Tris-borate-EDTA. For 2D TLC analysis, 2  $\mu\text{l}$  of the nuclease P1-digested products were applied directly to cellulose-coated TLC plates (EMD Biosciences) and developed in isobutyric acid: $\text{H}_2\text{O}$ :ammonium hydroxide (66:33:1; v:v:v) as a first dimension solvent and 0.1 M sodium phosphate (pH 6.8):ammonium sulfate:1-propanol (100:60:2; v:w:v) in the second-dimension solvent. Radioactive products were visualized and quantitated with a Storm 640 phosphorimager using ImageQuant software (Molecular Dynamics).

### Gel mobility shift assay

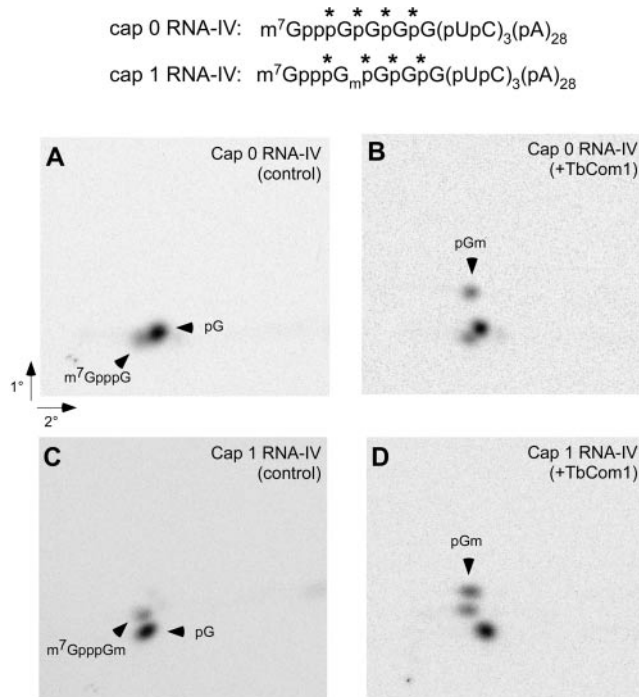
Standard binding mixtures (10  $\mu\text{l}$ ) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 5% glycerol, 1 pmol of  $^{32}\text{P}$ -labeled capped RNA and TbCom1 as specified were incubated at room temperature for 15 min then on ice for 15 min. Binding mixtures were applied to a 6% native polyacrylamide gel in 0.5 $\times$  Tris-borate-EDTA buffer and run at 75 V at  $4^{\circ}\text{C}$  until the bromophenol blue dye standard migrated ~50% the length of the gel. The complexes were detected by autoradiography of the dried gel and the extent of RNA-binding (bound/[bound + free]) was quantitated by phosphorimager.

## RESULTS

### Cap-dependent RNA methyltransferase activity of TbCom1

We expressed the TbCom1 protein in *E. coli* as a His<sub>10</sub>-tagged fusion and purified from a soluble extract by adsorption to Ni-agarose and phosphocellulose chromatography (Materials and Methods). To evaluate whether recombinant TbCom1 possesses cap-dependent RNA methyltransferase activity, we synthesized 35mer cap 0- and cap 1-terminated RNA substrates that contain radiolabeled 5'-phosphates only at the first four transcribed nucleosides, designated as cap 0 RNA-IV and cap 1 RNA-IV, respectively (Figure 2). Digestion of cap 0 RNA-IV with nuclease P1 followed by 2D TLC resulted in two radiolabeled spots corresponding to pG and  $\text{m}^7\text{GpppG}$  (Figure 2A). Note that the radioactivity of pG is derived from the 5'-phosphate of nucleosides 2, 3 and 4 and thus the intensity is 3-fold greater than that of  $\text{m}^7\text{GpppG}$ . We verified that the positions of these spots correspond to pG and  $\text{m}^7\text{GpppG}$  by Ultraviolet (UV) shadowing non-radioactive nucleoside 5'-pG and  $\text{m}^7\text{GpppG}$ . Similarly, digestion of cap 1 RNA-IV resulted in two radiolabeled spots, corresponding to pG and  $\text{m}^7\text{GpppGm}$  (Figure 2C). The  $\text{m}^7\text{GpppGm}$  migrated slightly faster than  $\text{m}^7\text{GpppG}$  in the first dimension due to methylation at position 1 of the transcribed RNA.

Incubation of recombinant TbCom1 with cap 0 RNA-IV in the presence of AdoMet followed by treatment with nuclease

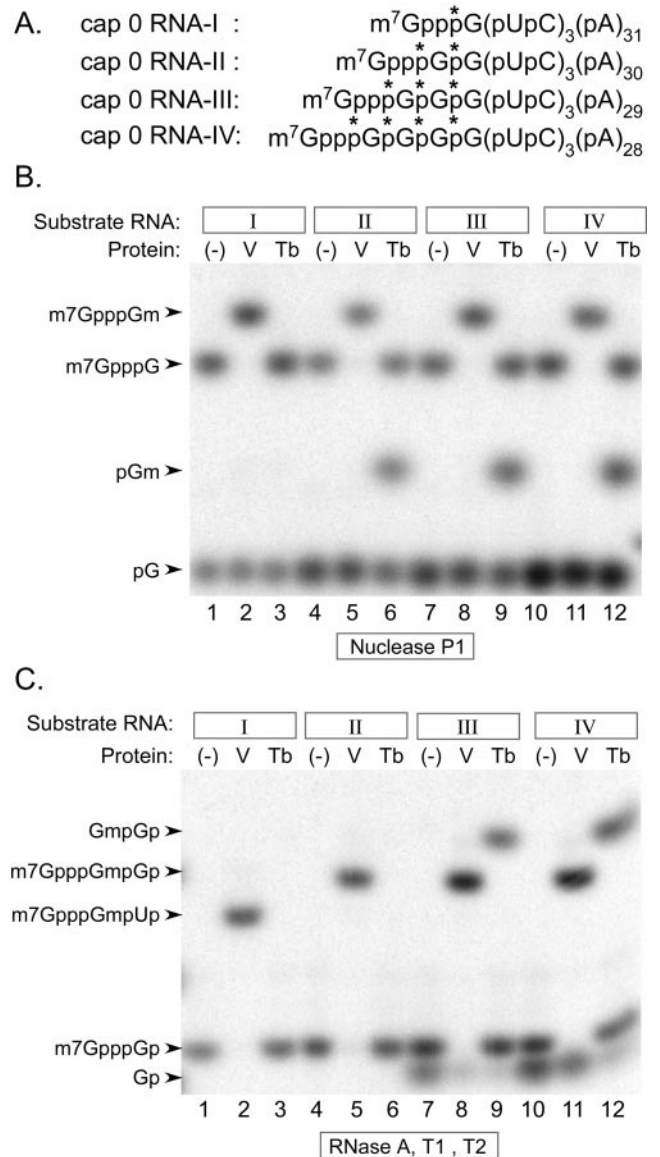


**Figure 2.** Analysis of modified nucleosides by two-dimensional thin layer chromatography. Reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 50 μM AdoMet and either 0.1 μM of <sup>32</sup>P-labeled cap 0 RNA-IV or cap 1 RNA-IV were incubated for 30 min at 27°C in the presence or absence of TbCom1. Mixtures were then digested with nuclease P1 and the products were separated by 2D TLC as described in Materials and Methods. (A) cap 0 RNA-IV alone; (B) cap 0 RNA-IV with 4 pmol of TbCom1; (C) cap 1 RNA-IV alone; (D) cap 1 RNA-IV with 4 pmol TbCom1. The positions of guanosine 5' monophosphate (pG), methylated guanosine 5' monophosphate (pGm), cap 0 dinucleoside (m<sup>7</sup>GpppG) and cap 1 dinucleoside (m<sup>7</sup>GpppGm) are indicated.

P1 resulted in the appearance of a new radiolabeled spot that migrated faster than pG in the first dimension (compare Figure 2A and B). Approximately one-third of the pG radioactivity was converted to the new radiolabeled species, whereas the radioactivity of m<sup>7</sup>GpppG remained constant. Based on the published 2D map of methylated guanosine nucleotides (23), we assigned this spot as pGm. An identical pGm spot was detected when cap 1 RNA-IV was incubated with TbCom1 (Figure 2D), suggesting that TbCom1 is not methylating the first transcribed nucleoside. We surmise that TbCom1 is capable of modifying either cap 0 or cap 1 RNA.

**TbCom1 methylates the 2nd position of transcribed RNA**

To determine the site of RNA methylation by TbCom1, we synthesized three additional cap 0 RNAs containing radiolabeled phosphates at positions 1, 2 and 3 of the transcribed RNA (designated cap 0 RNA-I, -II and -III, respectively; Figure 3A). TbCom1 was incubated with these capped RNAs in the presence of AdoMet and then treated with either nuclease P1 or an RNase cocktail. The digested products were resolved on a high percentage denaturing polyacrylamide gel. In the control reaction, digestion of cap 0 RNA-I, -II, -III or -IV with nuclease P1 generated m<sup>7</sup>GpppG (Figure 3B; lanes 1, 4, 7 and 10). Addition of VP39 prior



**Figure 3.** TbCom1 transfers a methyl group to the 2'-OH ribose moiety of the 2nd transcribed nucleoside. (A) Capped RNA substrates. <sup>32</sup>P-labeled 35mer cap 0 RNA substrates (RNA-I, -II, -III and -IV) were prepared by *in vitro* transcription as described in Materials and Methods. Asterisks indicate positions of <sup>32</sup>P-labeled phosphates. One pmol of <sup>32</sup>P-labeled cap 0 RNA-I, -II, -III and IV as shown in (A) was incubated in 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 50 μM AdoMet for 30 min at 27°C with either 4 pmol of vaccinia VP39 (V: lanes 2, 5, 8 and 11) or TbCom1 (Tb; lanes 3, 6, 9 and 12). Control reaction in the absence of methyltransferase are indicated by (-). Reaction products were digested with either nuclease P1 (B) or an RNase cocktail (C), and products were resolved by 22% denaturing polyacrylamide gel. The autoradiographs of the gels are shown.

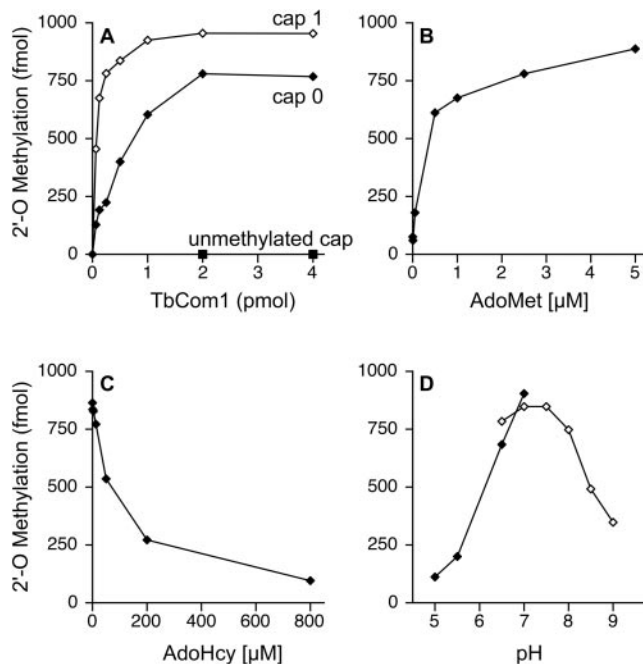
to nuclease P1 digestion shifts the m<sup>7</sup>GpppG to a slower-migrating band corresponding to the m<sup>7</sup>GpppGm cap 1 structure (Figure 3B; lanes 2, 5, 8 and 11). Incubation of TbCom1 with any of the cap 0 RNA substrates did not alter the mobility of m<sup>7</sup>GpppG (Figure 3B; lanes 3, 6, 9 and 12), consistent with the 2D TLC result that TbCom1 does not modify the nucleoside at position 1. When cap 0 RNA-II, -III or -IV was incubated with TbCom1, a novel radiolabeled species

appeared which migrated between  $m^7\text{GpppG}$  and  $p\text{G}$  (Figure 3B; lanes 6, 9 and 12). This radiolabeled species likely corresponds to methylated  $p\text{G}$  ( $p\text{Gm}$ ), as the appearance of this band was dependent on the addition of AdoMet (data not shown). Furthermore, treatment with alkaline phosphatase after nuclease P1 digestion eliminated this species (data not shown). The methyl transfer reaction was specific to cap 0- and cap 1-terminated RNA. No detectable methylation was observed using an RNA substrate lacking the cap N-7 methyl group (Figure 4A and data not shown).

To determine if modification occurs at the 2'-hydroxyl of the ribose sugar, identical reactions were digested with an RNase cocktail and the products were similarly resolved on a polyacrylamide gel (Figure 3C). Note that methylation at the 2'-hydroxyl of the ribose will prevent digestion by RNase resulting in the generation of a longer RNA fragment. In a control reaction, RNase cleaves all four cap 0 RNA substrates and generates  $m^7\text{GpppGp}$  (Figure 3C; lanes 1, 4, 7 and 10). The 3'-phosphate shifts the migration of the cap dinucleotide to a faster migrating band compared to  $m^7\text{GpppG}$ . Methylation at the first nucleoside by VP39 protects the RNA from digestion between nucleosides 1 and 2, producing

the cap 1 products  $m^7\text{GpppGmpUp}$  (Figure 3C, lane 2) or  $m^7\text{GpppGmpGp}$  (Figure 3C, lanes 5, 8 and 11). Note that the difference in mobility of the cap 1 products is due to the difference in nucleoside composition at position 2 (uracil versus guanine). As expected, incubation of TbCom1 with any of the cap 0 RNAs did not alter the mobility of  $m^7\text{GpppGp}$ , further confirming that methylation does not take place on the first transcribed nucleoside (Figure 3C, lanes 3, 6, 9 and 12). When TbCom1 was incubated with cap 0 RNA-III, a radiolabeled fragment which migrated slower than  $m^7\text{GpppGmpGp}$  was detected (Figure 3C, lane 9). We predict that this TbCom1 specific fragment corresponds to a methylated dinucleoside ( $\text{G}_{2\text{m}}\text{pG}_3\text{p}$ ), derived from nucleosides 2 and 3, that was resistant to RNase digestion due to 2'-O methylation. An identical radiolabeled product was detected when cap 0 RNA-IV was incubated with TbCom1 (Figure 3C, lane 12).

To address whether TbCom1 can methylate nucleosides other than guanosine, we substituted the guanosine at position 2 of the cap 0 RNA-IV with either U or C to obtain  $m^7\text{GpppGpUpCp}$  and  $m^7\text{GpppGpCpUp}$  RNAs. Incubation of TbCom1 with these substrates followed by treatment with RNase results in the formation of  $\text{C}_{2\text{m}}\text{pU}_3\text{p}$  and  $\text{U}_{2\text{m}}\text{pC}_3\text{p}$  dinucleotide products, respectively (data not shown). Furthermore, TbCom1 was capable of methylating the 2'-OH of the second transcribed nucleoside of either cap 0- or cap 1-terminated poly(A) (data not shown). Taken together, we surmise that TbCom1 is a cap-dependent, sequence-independent, 2'-O nucleoside methyltransferase that specifically methylates the 2nd position of a transcribed RNA.



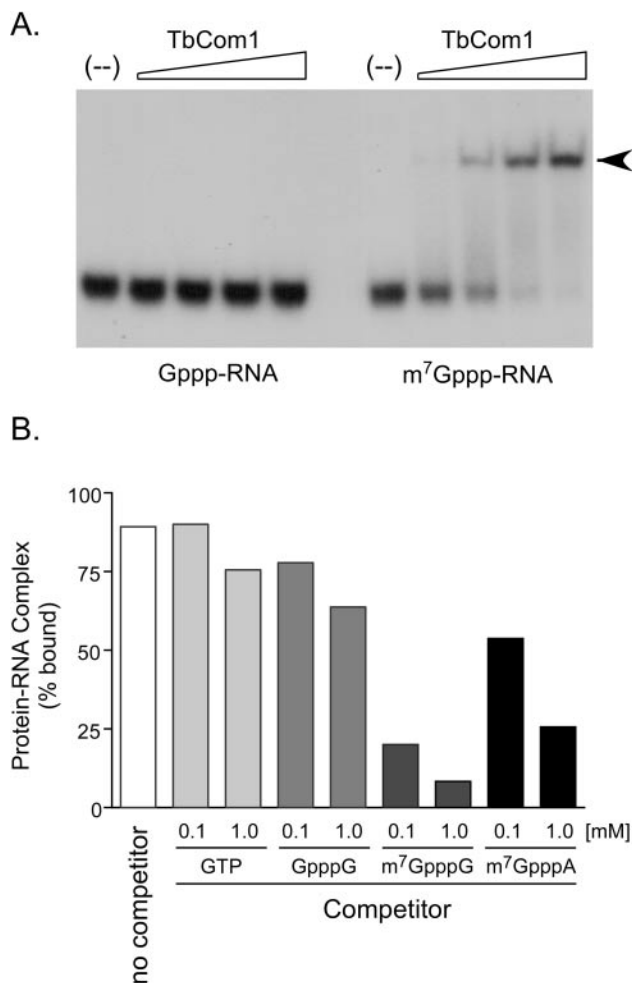
**Figure 4.** Characterization of TbCom1 RNA 2'-O methyltransferase activity. (A) Protein titration. Reaction mixtures (10  $\mu\text{l}$ ) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 50  $\mu\text{M}$  AdoMet, 1 pmol of either cap 1-, cap 0- or unmethylated cap RNA-IV, and TbCom1 as specified were incubated for 30 min at 27°C. Products were subjected to nuclease P1 digestion and analyzed by TLC. The extent of 2'-O methylation at position +2 is plotted as a function of input enzyme. (B) AdoMet dependence. Reaction mixtures (10  $\mu\text{l}$ ) contained 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.1  $\mu\text{M}$  of cap 0 RNA-IV, 4 pmol TbCom1, and AdoMet as indicated. Formation of  $p\text{Gm}$  is plotted as a function of AdoMet concentration. (C) Inhibition by AdoHcy. Reaction mixtures (10  $\mu\text{l}$ ) contained 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.1  $\mu\text{M}$   $m^7\text{GpppRNA}$ , 50  $\mu\text{M}$  AdoMet, 4 pmol TbCom1, with the indicated amounts of AdoHcy. The extent of methylation is plotted as a function of AdoHcy concentration. (D) pH dependency. Reaction mixtures (10  $\mu\text{l}$ ) containing 50 mM Tris buffer [either Tris-acetate (pH 5.0), 5.5, 6.5, 7.0 or Tris-HCl (pH 6.5, 7.0, 7.5, 8.0, 8.5, 9.0)], 5 mM DTT, 0.1  $\mu\text{M}$  of cap 0 RNA-IV and 4 pmol of TbCom1. The extent of 2'-O methylation is plotted as function of pH.

#### Characterization of TbCom1 2'-O methyltransferase activity

The extent of nucleoside 2'-O methylation on cap 0- and cap 1-RNA during a 30 min incubation at 27°C was proportional to input TbCom1 (Figure 4A). In the linear range of enzyme dependence, we estimate  $\sim 1.5$  pmol of cap 0 RNA can be methylated by 1 pmol of input enzyme in 30 min. The specific activity for methylating cap 1 RNA was  $\sim 5$ -fold higher compared to cap 0 RNA. TbCom1 was unable to transfer the methyl group to unmethylated capped RNA at the enzyme concentration sufficient to methylate the entire input cap 1 RNA. Methylation was dependent on AdoMet concentration from 0.1 to 0.5  $\mu\text{M}$  and the yield of methylated product plateaued above 1  $\mu\text{M}$  AdoMet (Figure 4B). Increasing concentration of AdoHcy in the reaction inhibited product formation;  $\sim 75\%$  inhibition was achieved at 200  $\mu\text{M}$  AdoHcy in the presence of 50  $\mu\text{M}$  AdoMet (Figure 4C). TbCom1 had a bell-shaped pH profile with optimal activity between pH 7.0–7.5 and activity declined sharply at acidic pH (Figure 4D).

#### Specificity of TbCom1 binding to $m^7\text{Gppp}$ -terminated RNA

To investigate whether TbCom1 specifically interacts with the cap structure, binding of TbCom1 to  $^{32}\text{P}$ -labeled cap 0 and unmethylated capped RNA was examined using a native gel mobility shift assay (Figure 5A). Binding reactions were performed in the absence of AdoMet to preclude conversion



**Figure 5.** TbCom1 binds specifically to m<sup>7</sup>Gppp-terminated RNA. (A) Gel mobility shift assay. Reaction mixtures (10  $\mu$ l) contained either 0.1  $\mu$ M of <sup>32</sup>P-labeled cap 0 RNA-IV (m<sup>7</sup>GpppRNA) or unmethylated cap RNA-IV (GpppRNA), with an increasing amount of TbCom1 (0.3, 0.6, 1.2 or 3.2 pmol, proceeding from left to right in the titration series). Control reactions without protein are indicated as (--). Products were analyzed by electrophoresis through a 6% native polyacrylamide gel. An autoradiogram of the dried gel is shown. The arrowhead to the right of the gel indicates protein-RNA complex. (B) Competition assays. Reaction mixtures contained 0.1  $\mu$ M of <sup>32</sup>P-labeled cap 0 RNA-IV, 1 pmol of TbCom1 and the indicated concentrations of either GTP, GpppG, m<sup>7</sup>GpppA or m<sup>7</sup>GpppG. Control reactions without protein are indicated as 'no competitor'. Protein-RNA complex formed was plotted as a function of cap analogue concentration.

of substrate to product during the incubation. Incubation of TbCom1 with the cap 0 RNA substrate resulted in the formation of a discrete protein-RNA complex that migrated more slowly than the free RNA (Figure 5A). The abundance of this complex increased in proportion to the amount of input enzyme, with an apparent dissociation constant of  $\sim$ 8 nM calculated based on (24). No specific complex was detected when TbCom1 was incubated with GpppRNA (Figure 5A). Thus, TbCom1 is capable of discriminating m<sup>7</sup>GpppN from GpppN at the substrate-binding step, confirming the importance of the N-7 methyl-guanine in substrate recognition.

To assess the binding properties of TbCom1, we performed competition assays with cap analogs. Either GTP, GpppG,

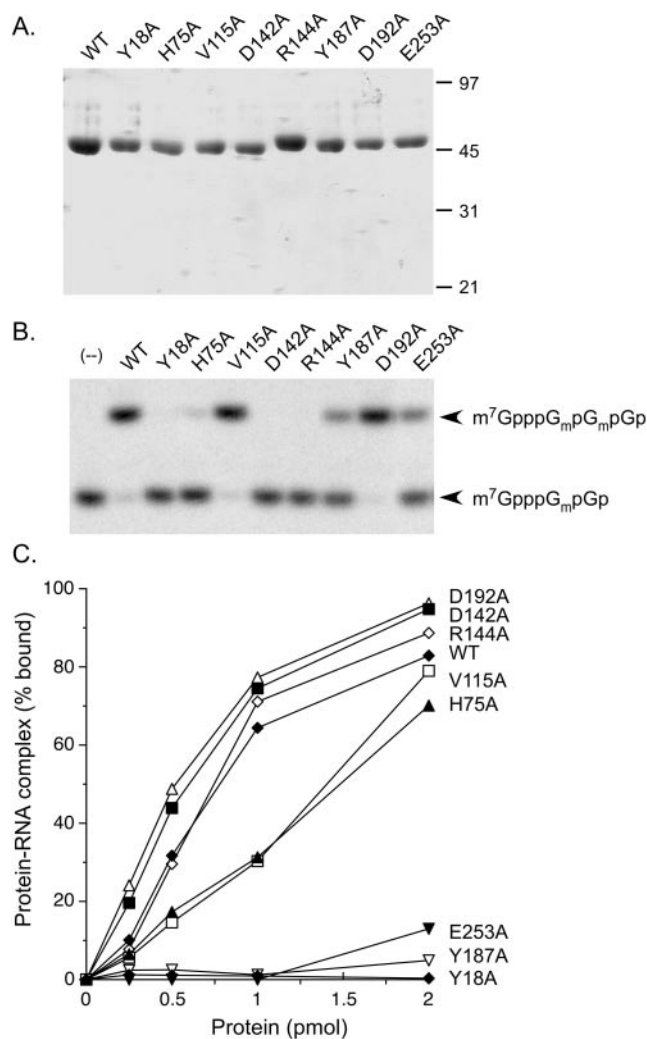
m<sup>7</sup>GpppG or m<sup>7</sup>GpppA at 0.1 mM or 1 mM was pre-incubated with 0.1  $\mu$ M <sup>32</sup>P-labeled cap 0 RNA, followed by addition of TbCom1. Neither GTP nor GpppG competed efficiently for binding by TbCom1 (Figure 5B). Both m<sup>7</sup>GpppG and m<sup>7</sup>GpppA competed for cap 0 RNA-binding by TbCom1, albeit only at high concentrations;  $\sim$ 80% inhibition was observed with 0.1 mM m<sup>7</sup>GpppG and 40% inhibition was observed with 0.1 mM m<sup>7</sup>GpppA. These results demonstrate that TbCom1 binds less avidly to cap analogues than to m<sup>7</sup>GpppG-terminated RNA and that the RNA chain is required for a stable interaction.

### Mutational analysis of TbCom1

We performed a mutagenesis analysis of TbCom1 to gain insight into the structural and functional requirements for the methyltransferase reaction. The structure of VP39 shows that the active site is comprised of two ligand-binding pockets: one for AdoMet and one for the m<sup>7</sup>G cap (17–19). The AdoMet-binding pocket in VP39 consists of a network of hydrogen bonds and van der Waals interactions with the AdoMet adenine (Phe115 and Val116), ribose (Asp95, Arg97, Val139 and Arg140) or methionine (Gln39, Tyr66, Gly72, His74 and Asp138). The essential m<sup>7</sup>G-binding pocket consists of two aromatic residues (Tyr22 and Phe180), which make base-stacking interactions with the guanine ring of the m<sup>7</sup>G cap. Additional hydrogen-bonding and van der Waals interactions with m<sup>7</sup>G are made by two acidic side-chains (Asp182 and Glu233) and by the backbone carbonyl of Tyr204. Of these 16 amino acids in VP39, 15 are either identical or conserved in TbCom1 (Figure 1), suggesting that TbCom1 may utilize a similar mechanism for recognition of AdoMet and capped RNA substrates as VP39, even though the enzymes differ in the positions they methylate on the RNA.

We selected eight residues in TbCom1 for alanine-substitution: three in the putative AdoMet-binding pocket (His75, Asp142 and Arg144) and four in the putative cap-binding pocket (Tyr18, Tyr187, Asp192 and Glu253) as indicated by asterisks in Figure 1. In addition, we mutated Val115, which is conserved among kinetoplastid Com1 proteins but is absent in VP39. The mutant TbCom1 proteins were expressed and purified in parallel with wild-type TbCom1 (Figure 6A), and their methyltransferase activities were compared to wild-type in the presence of 50  $\mu$ M AdoMet and 0.1  $\mu$ M cap 1-terminated RNA-IV (Figure 6B). The Y18A, H75A, D142A and R144A mutations reduced the methyltransferase activity to  $<$ 2% of wild-type level. The Y187A and E253A proteins showed moderately reduced levels of activity (16 and 22% of wild-type level, respectively). In contrast, the V115A and D192A mutations retain near wild-type activities.

To evaluate the nature of the defect in these mutant proteins, their binding affinities to m<sup>7</sup>Gppp-terminated RNA substrates were measured by gel mobility shift assay (Figure 6C). The Y18A, Y187A and E253A mutations show significant reductions in binding to the capped RNA. By comparing the extent of binding at equivalent concentrations of proteins, we estimated that Y18A was reduced to 1% of the wild-type level. The Y187A and E253A mutants had 5–10% of wild-type binding. The H75A and V115A mutants



**Figure 6.** Effects of alanine-substitution on TbCom1 methyltransferase activity. (A) Protein purification. Aliquots (3  $\mu$ g) of phosphocellulose fractions of wild-type (WT) or the indicated alanine-mutants were analyzed by SDS-PAGE. Polypeptides were visualized with Coomassie blue dye. The positions and sizes (in kDa) of co-electrophoresed marker polypeptides are indicated to the right. (B) Cap methyltransferase assays. Reaction mixtures (10  $\mu$ l) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 50  $\mu$ M AdoMet, 0.1  $\mu$ M  $^{32}$ P-labeled cap 1 RNA-IV and 4 pmol of wild-type (WT) or mutant TbCom1. After incubation for 30 min at 27°C, samples were digested with RNase cocktail and products were resolved on a 22% denaturing polyacrylamide gel. An autoradiogram of the gel is shown. Labeled species corresponding to m<sup>7</sup>GpppGmpGp (cap 1) and m<sup>7</sup>GpppGmpGmpGp (cap 2) are indicated at the left. (C) Protein-RNA complex formation. The extent of capped RNA-binding by wild-type and mutant TbCom1 proteins were analyzed by gel mobility shift assay. Percentage of protein-RNA complex formed was plotted as a function of input protein.

had slightly reduced binding ( $\sim$ 50% of the wild-type level), whereas the D142A, R144A and D192A proteins maintained strong binding to capped RNA. Thus the aromatic side-chains (Tyr18 and Try187) and the acidic side-chain (Glu253) are essential for recognition of capped RNA.

## DISCUSSION

The mRNAs of kinetoplastid protozoans possess a unique cap 4 structure on the SL RNA, which is transferred to the 5' end

of all mRNAs by trans-splicing (6,11). While the enzymes that catalyze cap 0 formation in *T.brucei* have been characterized (25–27), the 2'-O nucleoside methyltransferase activities that lead to cap 4 have not been described. *T.brucei* encodes 48 and 57 kDa proteins with similarity to the vaccinia VP39 cap-dependent 2'-O nucleoside RNA methyltransferase (14). We showed that the 48 kDa recombinant TbCom1 protein transfers a methyl group from AdoMet to m<sup>7</sup>GpppN-terminated RNA at the 2'-OH position of the second nucleoside of the transcribed RNA to form m<sup>7</sup>GpppNpNm. TbCom1 is capable of methylating either cap 0- or cap 1-terminated RNA, indicating that methylation at the second nucleoside occurs after cap 0 formation but can take place prior to methylation at the first nucleoside. TbCom1 is the only cellular cap-dependent 2'-O methyltransferase that has been characterized biochemically with a unique specificity for methylating the second nucleoside of the mRNA.

TbCom1 binds stably to a 35mer m<sup>7</sup>GpppN-terminated RNA, whereas like VP39, no complex was formed with GpppN-terminated RNA (28). Thus TbCom1 is capable of discriminating methylated versus unmethylated capped RNA at the substrate-binding step. The apparent dissociation constant of TbCom1 binding to methylated capped RNA was about 8 nM, a value comparable to the affinity of VP39 with 50mer m<sup>7</sup>GpppN-RNA [ $K_m = 9.3$  nM (28)]. We note that m<sup>7</sup>GpppA and m<sup>7</sup>GpppG cap analogues are weak competitors of TbCom1 binding to m<sup>7</sup>GpppN-RNA, suggesting that the enzyme requires a downstream RNA chain for stable interaction. The fact that TbCom1 can bind to and methylate RNA in a non-sequence dependent manner implies that 2'-O methylation can take place on RNAs other than the SL RNA, such as snRNAs and polycistronic pre-mRNAs, although it is not known whether polycistronic pre-mRNAs are capped. Alternatively, an *in vivo* specificity for cap 4 modification to SL RNA is achieved by the recruitment of methyltransferase components to the site of SL RNA transcription, as cap 4 formation appears to take place cotranscriptionally during SL RNA synthesis (7).

We identified 6 amino acids (Tyr18, His75, Asp142, Arg144, Tyr187 and Glu253) that are essential or important for TbCom1 methyltransferase activity. Alanine-substitution at His75, Asp142 and Arg144 in the putative AdoMet-binding motif severely reduces TbCom1 methyltransferase activity, but the mutant proteins retain the ability to bind to capped RNA. Based on the crystal structure of VP39 complexed with AdoMet, TbCom1 His75 and Asp142 (equivalent of VP39 His74 and Asp138, respectively) may form hydrogen bonds with the terminal oxygen atoms of the AdoMet methionine. The Arg144 in TbCom1 may interact with the backbone nitrogen atom of the ribose ring of AdoMet, analogous to Arg140 in VP39. Consistent with our results, the D138A-R140A double mutant in VP39 was deficient for AdoMet-binding and methyltransferase activity (29,30).

In addition to the VP39 structure, insight into the mechanism of m<sup>7</sup>G cap-recognition has been gained from the atomic structures of human nuclear cap-binding complex (CBC) and eIF4E complexed with either m<sup>7</sup>GDP or methylated cap analogues (31–33). While the overall structures of these proteins differ significantly, the guanine ring of the

cap nucleotide is sandwiched by base-stacking interactions with two aromatic residues in all three proteins: Tyr18/Phe180 in VP39, Trp56/Trp102 in eIF4E and Tyr20/Try43 in CBP20 subunit of CBC. Perturbation of the aromaticity by mutagenesis of each of these proteins decreases the affinity, and in some cases the specificity, for the cap structure (19,33,34). We propose that TbCom1 utilizes analogous base-stacking interactions for recognition of the m<sup>7</sup>G moiety, as mutation of the equivalent aromatic residues (Tyr18 and Tyr187) led to reduced methyltransferase activity and abolished capped RNA-binding. It is noteworthy that the Y18A mutation had a more severe effect on binding to and methylation of capped RNA than the Y187A mutation. This is in contrast to VP39, where alanine substitution at the first aromatic residue (Y22A) had a less dramatic effect on methyltransferase activity than alanine-substitution at the second aromatic residue (F180A) (19).

In addition to the base-stacking interactions, at least one acidic side-chain in TbCom1 is essential for binding to capped RNA. Alanine-substitution of Glu253 in TbCom1 led to a reduction in methyltransferase activity and cap-specific binding. The corresponding acidic residues in VP39 (Glu233), eIF4E (Glu103) and the CBP20 subunit (Asp116) interact with the N1 and/or N2 atom of the m<sup>7</sup>G purine ring via salt-bridges or hydrogen bonds (19,20,33–36). Removal of these acidic side-chains decreased cap-specific binding in all three proteins. We predict that the carboxyl side-chain of Glu253 in TbCom1 interacts with the positively charged m<sup>7</sup>G moiety to enhance cap specificity. The Asp192 of TbCom1 corresponds to Asp182 of VP39. The Asp182 side-chain in VP39 forms a bi-dentate hydrogen bond with the N2 atom of the cap guanine (19). However, the VP39 D182A mutation had no effect on methyltransferase activity or binding to capped RNA. Similarly, we found that the TbCom1 D192A mutant had wild-type methyltransferase activity and cap-specific binding. Thus, it appears that the asparagine side-chain does not significantly contribute to binding to the m<sup>7</sup>G cap or catalysis in either VP39 or TbCom1.

While our biochemical analysis of TbCom1 was in progress, genetic knockouts and RNAi-mediated knockdowns of the 48 kDa *TbCOM1* and 57 kDa *T.brucei* genes were generated (which were called *TbMT48* and *TbMT57*, respectively) (37–39). Neither the *TbCOM1/TbMT48* nor *TbMT57* knockouts, or the simultaneous knockdowns of both genes, in procyclic *T.brucei* had effects on viability or trans-splicing. Consistent with our biochemical data, *TbCOM1/TbMT48*-deficient cell lines lacked 2'-O-ribose methylation at position +2 on the SL RNA (38,39). In contrast, SL RNA isolated from *TbMT57* deficient cell lines accumulated cap 2, implying that *TbMT57* is involved in methylation at positions +3 and/or +4 on the SL RNA (37,39). However, the enzymatic activity of the *TbMT57* gene product has not been characterized and thus it is not clear what is the substrate requirement for its methyltransferase activity. Further structural and functional studies of TbCom1 and the *TbMT57* gene product, as well as identification of the methyltransferases responsible for SL RNA base modifications, should provide a better understanding of how cap 4 modifications are mechanistically coordinated and the role of cap 4 in kinetoplast parasite gene expression.

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