Biochemical and genetic analysis of RNA cap guanine-N2 methyltransferases from Giardia lamblia and Schizosaccharomyces pombe

Stéphane Hausmann¹, Alejandro Ramirez², Susanne Schneider², Beate Schwer² and Stewart Shuman¹,*

¹Molecular Biology Program, Sloan-Kettering Institute, New York, NY 10021, USA and ²Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY 10021, USA

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ABSTRACT

RNA cap guanine-N2 methyltransferases such as Schizosaccharomyces pombe Tgs1 and Giardia lamblia Tgs2 catalyze methylation of the exocyclic N2 amine of 7-methylguanosine. Here we performed a mutational analysis of Giardia Tgs2, entailing an alanine scan of 17 residues within the minimal active domain. Alanine substitutions at Phe18, Thr40, Asp76, Asn103 and Asp140 reduced methyltransferase specific activity to 5–3% of wild-type Tgs2, thereby defining these residues as essential. Alanines at Pro142, Tyr148 and Pro185 reduced activity to 7–12% of wild-type. Structure–activity relationships at Phe18, Thr40, Asp76, Asn103, Asp140 and Tyr148, and at three other essential residues defined previously (Asp68, Glu91 and Trp143) were gleaned by testing the effects of 18 conservative substitutions. Our results engender a provisional map of the Tgs2 active site, which we discuss in light of crystal structures of related methyltransferases. A genetic analysis of S. pombe Tgs1 showed that it is nonessential. An S. pombe tgs1/C² strain grows normally, notwithstanding the absence of 2,2,7-trimethylguanosine caps on its U1, U2, U4 and U5 snRNAs. However, we find that S. pombe requires cap guanine-N7 methylation catalyzed by the enzyme Pcm1. Deletion of the pcm1¹ gene was lethal, as were missense mutations in the Pcm1 active site. Thus, whereas m7G caps are essential in both S. pombe and S. cerevisiae, m²,²,²G caps are not.

INTRODUCTION

The 5’ m7GpppN cap is a distinctive feature of eukaryotic viral and cellular mRNA. Cap synthesis entails three enzymatic reactions: (i) the 5’ triphosphate end of the pre-mRNA is hydrolyzed to a diphosphate by RNA trypophosphatase; (ii) the diphosphate RNA end is capped with GMP by RNA guanylyltransferase; and (iii) the GpppN cap is methylated by RNA (guanine-N7) methyltransferase (1). RNA guanylyltransferase is essential for cell growth in all organisms where its function has been tested, including: the fungi Saccharomyces cerevisiae (2–4), Schizosaccharomyces pombe (5) and Candida albicans (6); the nematode Caenorhabditis elegans (7,8); and cultured human cells (9). Cap guanine-N7 methyltransferase is essential for the viability of S. cerevisiae (10–13), but is reported to be nonessential in C. albicans (6).

A subset of capped RNAs contain one or two additional methyl groups attached to the exocyclic N2 of the cap guanosine. A 2,2,7-trimethylguanosine (TMG) cap is found on many small noncoding eukaryal RNAs such as small nuclear (sn) and small nucleolar (sno) RNAs and telomerase RNA (14,15) and on nematode mRNAs that undergo trans-splicing of a 5’-capped leader sequence (16). A 2,7-dimethylguanosine (DMG) cap is detected in the mRNAs of two RNA viruses: Sindbis virus and Semliki Forest virus (17,18). TMG synthesis has been of considerable interest to RNA biologists because of the involvement of snRNAs in pre-mRNA splicing (19–22). A breakthrough in defining the genetic pathway of TMG cap formation was made in 2002 when Remy Bordonnè and colleagues identified the Schizosaccharomyces pombe Tgs1 protein in an interaction screen using a yeast Sm protein as bait (23). The presence of a putative

*To whom correspondence should be addressed. Email: s-shuman@ski.mskcc.org

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AdoMet binding motif in the Tgs1 polypeptide, mutation of which affected TMG formation in vivo (23,24), suggested that Tgs1 might be directly involved in TMG formation. Our biochemical studies of Schizosaccharomyces pombe Tgs1 showed that it is indeed the agent of TMG synthesis (25). Tgs1 catalyzes methyl transfer from AdoMet to m7GTP, m7GDP or m7GpppA (but not GTP, GDP, GpppA). The m7GDP product formed by Tgs2 corresponding to 66VIDGTACVGG75 and 88VAIE91 in Tgs2. A binding site for the methyl acceptor was predicted by Mouaik et al. (24) to be composed of two conserved peptides, corresponding to 66VIDGTACVGG75 and 88VAIE91 in Tgs2. A binding site for the methyl acceptor was predicted to reside within a proline/glycine-containing peptide, corresponding to 140DPPWGGV146 in Tgs2. Bordonné and colleagues showed that Ala mutations of S. cerevisiae Tgs1 at three positions in these motifs (Asp103, Asp126 and Trp178, corresponding to Asp68, Glu91 and Trp143 in Tgs2) caused defects in TMG cap formation in vivo (23,24). To gauge the biochemical effects of such changes, we previously produced and purified Giardia Tgs2 mutants D68A, E91A and W143A. These proteins were inert in catalysis of methyl transfer from AdoMet to m7GDP (26). Based on the crystal structure of cap guanine-N7 methyltransferase bound to AdoMet and mutational analysis of that enzyme (13,27), we proposed that Tgs-like proteins from diverse sources have similar primary structures, as illustrated in Figure 1, in which the sequence of Giardia Tgs2 is aligned to the sequence of a second Giardia paralog (Tgs1) and to the sequences of Tgs1 of S. pombe, S. cerevisiae and Homo sapiens. An AdoMet-binding site was predicted by Mouaike et al. (24) to be composed of two conserved peptides, corresponding to 66VIDGTACVGG75 and 88VAIE91 in Tgs2. A binding site for the methyl acceptor was predicted to reside within a proline/glycine-containing peptide, corresponding to 140DPPWGGV146 in Tgs2. Bordonné and colleagues showed that Ala mutations of S. cerevisiae Tgs1 at three positions in these motifs (Asp103, Asp126 and Trp178, corresponding to Asp68, Glu91 and Trp143 in Giardia Tgs2) caused defects in TMG cap formation in vivo (23,24). To gauge the biochemical effects of such changes, we previously produced and purified Giardia Tgs2 mutants D68A, E91A and W143A. These proteins were inert in catalysis of methyl transfer from AdoMet to m7GDP (26). Based on the crystal structure of cap guanine-N7 methyltransferase bound to AdoMet and mutational analysis of that enzyme (13,27), we proposed that Asp68 and Glu91 coordinate the methionine amine and adenosine ribose hydroxyls, respectively (26). Here we conducted a more extensive biochemical structure–function analysis of Giardia Tgs2, including: (i) characterization of N- and C-terminal truncation mutants to delineate a minimal domain capable of methyltransferase activity in vitro and (ii) an alanine scan of conserved residues to identify individual essential side chains. The results of the alanine-scan engendered methyltransferase superfamily, will rely on structural motifs common to all superfamily members (e.g. for AdoMet binding), while exploiting novel structural determinants of mG methyl acceptor specificity. Tgs-like proteins from diverse sources have similar primary structures, as illustrated in Figure 1, in which the sequence of Giardia Tgs2 is aligned to the sequence of a second Giardia paralog (Tgs1) and to the sequences of Tgs1 of S. pombe, S. cerevisiae and Homo sapiens. An AdoMet-binding site was predicted by Mouaike et al. (24) to be composed of two conserved peptides, corresponding to 66VIDGTACVGG75 and 88VAIE91 in Tgs2. A binding site for the methyl acceptor was predicted to reside within a proline/glycine-containing peptide, corresponding to 140DPPWGGV146 in Tgs2. Bordonné and colleagues showed that Ala mutations of S. cerevisiae Tgs1 at three positions in these motifs (Asp103, Asp126 and Trp178, corresponding to Asp68, Glu91 and Trp143 in Giardia Tgs2) caused defects in TMG cap formation in vivo (23,24). To gauge the biochemical effects of such changes, we previously produced and purified Giardia Tgs2 mutants D68A, E91A and W143A. These proteins were inert in catalysis of methyl transfer from AdoMet to m7GDP (26). Based on the crystal structure of cap guanine-N7 methyltransferase bound to AdoMet and mutational analysis of that enzyme (13,27), we proposed that Asp68 and Glu91 coordinate the methionine amine and adenosine ribose hydroxyls, respectively (26). Here we conducted a more extensive biochemical structure–function analysis of Giardia Tgs2, including: (i) characterization of N- and C-terminal truncation mutants to delineate a minimal domain capable of methyltransferase activity in vitro and (ii) an alanine scan of conserved residues to identify individual essential side chains. The results of the alanine-scan engendered...
a round of conservative mutagenesis to determine structure–activity relationships at nine positions.

A major surprise accompanying the initial identification of *S. cerevisiae* Tgs1 was that a *tgs1* deletion mutant was viable, even though the snRNAs and snoRNAs in the *tgs1Δ* strain lacked TMG caps (23). A pertinent question is whether other fungi can also survive without TMG caps. We addressed this question by deleting the *S. pombe* *tgs1* gene. It is not a foregone conclusion that dispensability of an RNA processing factor in *S. cerevisiae* can be extrapolated to *S. pombe*. For example, the pre-mRNA splicing factor Prp18 is not essential in *S. cerevisiae*, but is essential in *S. pombe* (B. Schwer, unpublished data). Also, deleting the intron lariat debranching enzyme Dbr1 is benign in *S. cerevisiae*, but elicits a severe growth defect in *S. pombe* (28). We report here that an *S. pombe* *tgs1Δ* strain grows normally, despite the absence of TMG caps on its snRNAs. However, we find that *S. pombe* requires cap guanine-N7 methylation, catalyzed by the enzyme Pcm1 (29), insofar as deletion of the *pcm1* gene is lethal, as are missense mutations in the active site of Pcm1.

**METHODS**

*Tgs2* deletion mutants

N-terminal deletion mutants *Tgs2-(14-258)* and *Tgs2-(31-258)* were constructed by PCR amplification with sense-strand primers that introduced a BglII restriction site at the Met14 and Met31 codons, respectively, and an antisense-strand primer that introduced a XhoI site 3′ of the stop codon. C-terminal deletions *Tgs2-(1-235)* and *Tgs2-(1-216)* were constructed by PCR amplification with mutagenic antisense primers that introduced stop codons in place of the codons for Ala236 or Arg217 and a XhoI site 3′ of the new stop codon. The PCR products were digested with BglII and XhoI and then inserted between BaumH1 and XhoI sites in the plasmid pET28-His10Smt3, so as to fuse the truncated Tgs2 proteins in-frame with an amino-terminal His10Smt3 domain. The plasmid inserts were sequenced completely to exclude the acquisition of unwanted mutations during amplification and cloning.

*Tgs2* missense mutants

Alanine and conservative mutations were introduced into the *TGS2* gene by PCR amplification with mutagenic primers. The mutated open reading frames were inserted between the SacI and XhoI sites of a customized bacterial expression vector pET28-His10Smt3m (a derivative of pET28-His10Smt3 which has a unique SacI site downstream of the Smt3 cassette). The inserts were sequence completely to verify that no unwanted coding changes had been introduced.

Recombinant Tgs2

The pET28-His10Smt3-Tgs2 plasmids were transformed into *E. coli* BL21-CodonPlus(DE3). Cultures (500 ml) derived from single transformants were grown at 37°C in LB medium containing 50 μg/ml kanamycin and 50 μg/ml chloramphenicol until the A600 reached 0.6. The cultures were adjusted to 0.2 mM IPTG and 2% ethanol and incubation was continued for 20 h at 17°C. Cells were harvested by centrifugation and stored at −80°C. All subsequent procedures were performed at 4°C. thawed bacteria were resuspended in 25 ml of buffer A (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol). PMSF and lysozyme were added to final concentrations of 500 μM and 100 μg/ml, respectively. After incubation on ice for 30 min, imidazole was added to a final concentration of 5 mM and the lysate was sonicated to reduce viscosity. Insoluble material was removed by centrifugation. The soluble extracts were mixed for 30 min with 1.6 ml of Ni²⁺-NTA-agarose (Qiagen) that had been equilibrated with buffer A containing 5 mM imidazole. The resins were recovered by centrifugation, resuspended in buffer A containing 5 mM imidazole, and poured into columns. The columns were washed with 10 ml of 20 mM imidazole in buffer A and then eluted step-wise with 2.5 ml aliquots of buffer A containing 50, 100, 250 and 500 mM imidazole. The elution profiles were monitored by SDS-PAGE. The 250 mM imidazole eluates containing the recombinant Tgs2 polypeptides were diaлизed against buffer containing 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 2 mM DTT, 1 mM EDTA, 10% glycerol and then stored at −80°C. The protein concentration was determined using the Bio-Rad dye binding method with BSA as the standard. Alternatively, the concentrations of some mutants were determined by SDS-PAGE analysis of the Tgs2 preparations in parallel with serial dilutions of a BSA standard. The gels were stained with Coomassie Blue, and the staining intensities of the Tgs2 and BSA polypeptides were quantified using a FujiFilm FLA-5000 digital imaging and analysis system. Tgs2 concentrations were calculated by interpolation to the BSA standard curve.

**Methyltransferase assay**

Reaction mixtures containing 50 mM Tris-HCl, pH 8.0, 5 mM DTT, 2.5 mM m′GDP, 50 μM [³H]-AdoMet, and enzyme were incubated for 15 min at 37°C. Aliquots (4 μl) were spotted on PEI-cellulose TLC plates, which were developed with 50 mM ammonium sulfate. The AdoMet- and m²,7GDP-containing portions of the lanes were cut out and the radioactivity in each was quantified by liquid scintillation counting.

**Gene disruptions in *S. pombe***

We produced *tgs1Δ* and *pcm1Δ* gene disruption cassettes using a modified version of the long flanking homology PCR technique (30). The 5′ flanking DNA segments (360 and 380-bp upstream of the translation start codons of *tgs1* and *pcm1*, respectively) were PCR-amplified from genomic DNA and inserted upstream of the *kanMX* gene in plasmid pFA6a-kanMX4. Then, 3′ flanking DNA segments (520 and 480-bp downstream of the stop codons for *tgs1* and *pcm1*, respectively) were PCR-amplified from genomic DNA and inserted downstream of the *kanMX* gene. The *tgs1::kanMX*
and pcm1::kanMX cassettes were excised by digestion with PvuII and SacII and used to transform a diploid strain of S. pombe.

The S. pombe diploid strain was generated by crossing two heterothallic strains FY527(ura4-D18 leu1-32 ade6-M216 his3-D1 h²) and FY528(ura4-D18 leu1-32 ade6-M210 his3-D1 h¹) on ME plates at room temperature. After 24 h, the cells were streaked onto medium lacking adenine to select for diploids. The Ade⁺ diploids were verified by staining with phloxin B and a single diploid colony was picked and incubated in 100 ml of YES medium to prepare competent S. pombe cells. The transformations were performed using the lithium acetate method (31). The integrants were selected at 30°C on YES plates containing 200 μg/ml G418. Single colonies were restreaked on YES agar containing G418. Genomic DNA was prepared from individual isolates and the integration of the tgs1::kanMX or pcm1::kanMX cassettes into the correct locus was tested by PCR using diagnostic primers and confirmed by Southern blotting. The heterozygous diploids were sporulated on ME plates at room temperature. Tetrads were dissected from single asci and zygous diploids were sporulated on ME plates at room temperature. The Ade⁺ diploids were tested for growth on YES agar and YES agar containing 50% of the input RNA, 100% of the bead-bound RNA and 60% of the supernatant RNA) were analyzed by electrophoresis through a 6% polyacrylamide-urea gel. The gel contents were transferred to a Hybond membrane. Specific RNAs were detected by northern blotting, entailing sequential probing with 5' 32P-labeled DNA oligonucleotides complementary to S. pombe U1 snRNA (5'-GCTGCAGAATTCTAGGGCTGTAAGT), U2 snRNA (5'-GAAAGATACTAATTCTTGC), U4 snRNA (5'-GGTTGGAGCCGGTACGTTAATAG), U5 snRNA (5'-GATTACAAAAACTACGTCACAATTTGAC) and 5.8S RNA (5'-CTTATCGATGCGAGGCAGAGATCG). The hybridized probes were detected by autoradiography and quantified with a phosphorimager.

**Pcm1 expression vectors**

The S. cerevisiae expression plasmid pYN-Pcm1(6-389), in which S. pombe pcm1⁰⁺ is under the transcriptional control of the TPI1 promoter (29) was digested with NdeI; the overlap was filled in with T4 DNA polymerase and the DNA was then digested with BamHI. The pcm1⁰⁺ fragment was gel-purified and ligated into the S. pombe expression vector pREP81x (containing the selectable LEU2 gene), which had been digested with XhoI, filled in with T4 DNA polymerase, and then digested with BamHI. In the resulting pREP81x-Pcm1 plasmid, pcm1⁰⁺ is under the transcriptional control of the nmt** promoter (34). The pcm1⁰⁺ DNA fragment was also inserted into pDS472 (containing S. pombe ura4⁺ gene) to create pDS472-Pcm1, in which pcm1⁰⁺ is driven by the nmt promoter (35). Missense mutations in pcm1⁰⁺ were introduced via the two-stage overlap PCR method using pYN-Pcm1(6-389) as the template. The second-stage PCR products were digested with NdeI and BamHI and inserted into pYN32 to generate the S. cerevisiae expression plasmids pYN-Pcm1-D145A, pYN-Pcm1-R181A and pYN-Pcm1-Y225A. The pcm1⁰⁺ inserts were sequenced completely to confirm the intended mutations and the absence of unintended coding changes. The mutated pcm1⁰⁺ genes were subcloned into the S. pombe pREP81x vector as described above for the wild-type gene. The S. cerevisiae ABD1 gene was excised from p358-ABD1 and inserted into pREP81x using the same strategy.

**Test of pcm1⁰⁺ function in S. pombe by plasmid shuffle**

The S. pombe pcm1⁰⁺ pcm1::kanMX diploid strain was transformed with plasmid pDS472-Pcm1 (pcm1⁰⁺ ura4⁰⁺). Ura⁺ KanR haploids containing the pcm1::kanMX chromosomal locus and the plasmid-borne pcm1⁺ allele were recovered after sporulation. The pcm1Δ pDS472-Pcm1 isolates were unable to grow on agar medium containing 1 mg/ml 5-fluoroorotic acid (FOA), a drug that selects against the ura4⁰⁺ plasmid. The pcm1Δ pDS472-Pcm1 strain was transformed with pREP81x (LEU2) plasmids bearing wild-type pcm1⁺, mutant alleles D145A, R181A and Y225A, or wild-type S. cerevisiae ABD1. A control transformation was performed with the

**RNA isolation and TMG analysis**

Schizosaccharomyces pombe tgs1⁺ and tgs1Δ strains were grown in YES medium until A₆₀₀ reached 1.0. Cells were harvested by centrifugation and washed with cold water. The washed cell pellets were stored frozen at −80°C prior to RNA isolation. Total cellular RNA was isolated using the hot-phenol method (32). TMG-containing RNA was immunoprecipitated with anti-TMG cap antibody R1131 (33; a generous gift of Dr R. Lührmann) as follows. Aliquots of the R1131 antibody (10 μg) were mixed with protein A sepharose beads [100 μl of a 50% slurry (w/v) in IPP-150 buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Nonidet-P40)] and gently mixed at 4°C for 2 h. The beads were washed by 3 cycles of centrifugation and resuspended in 500 μl of cold IPP-150. Total S. pombe RNA (5 μg) was added to the bead pellet in 200 μl of IPP-150. The samples were mixed gently for 2 h at 4°C, then the supernatant was removed and the unbound RNA in the supernatant was recovered by phenol–chloroform extraction and ethanol precipitation. The bead pellet was washed three times with 500 μl cold IPP-150. The beads were then suspended in 200 μl IPP-150. The bead-bound RNA was extracted twice with phenol-chloroform and concentrated by ethanol precipitation. Aliquots of the
empty pREP81x vector. Transformants were selected on minimal medium lacking leucine (36). Individual Leu<sup>+</sup> isolates were then streaked to agar plates containing 1 mg/ml FOA. Only strains containing a plasmid encoding a biologically active cap guanine-N<sup>7</sup> methyltransferase will give rise to FOA-resistant colonies.

RESULTS AND DISCUSSION

Mapping the Tgs2 active site by mutagenesis

We initially sought to delineate a minimal functional domain of the 258-aa Tgs2 polypeptide by testing the effects of incremental deletions from the N- and C-termini. The full-length Tgs2 and truncated proteins Tgs2-(1-235), Tgs2-(14-258) and Tgs2(31-258) were produced in E. coli as N-terminal His<sub>10</sub>-Smt3 fusions and purified from soluble extracts by Ni-agarose chromatography (Figure 2A). Enzyme titrations in the presence of saturating substrate concentrations (2.5 mM m<sup>7</sup>GDP; 50 μM AdoMet) showed that Tgs2-(1-235) was as active as full-length Tgs2, signifying that the C-terminal 23-aa were dispensable for catalysis in vitro (Figure 2B). This result led us to construct a further C-terminal truncation, Tgs2-(1-216), but this derivative was insoluble when produced in bacteria (not shown). Whereas the first N-terminal truncation Tgs2-(14-258) retained about one-fifth of the wild-type activity, the next deletion, Tgs2(31-258) was catalytically inert (Figure 2B). We surmise that the segment between positions 15 and 30 either includes one or more critical functional groups or is needed for proper folding of the Tgs2 protein.

Guided by these results, we introduced single Ala mutations into the full-length Tgs2 protein at the 17 amino acids indicated by filled circles above the Tgs2 sequence in Figure 1. The targeted residues are located within the minimal domain and most are conserved among the Tgs homologs. The His<sub>10</sub>-Smt3-Tgs2-Ala proteins were produced in bacteria and purified by Ni-agarose chromatography (Figure 3A). Enzyme titrations revealed that 14 of the alanine mutants displayed detectable cap guanine-N2 methyltransferase activity and that the extent of m<sup>2</sup>7GDP formation was proportional to input enzyme (Figure 4). Three of the mutants—T40A, N103A and D140A—had no activity at up to 0.5 μg of input protein (Figure 4). The specific activities of the Tgs2-Ala mutants were calculated from the slopes of the titration curves and normalized to the wild-type specific activity. The results are compiled in Table 1.

We operationally defined an important side chain as one at which Ala substitution reduced specific activity to <15% of the wild-type value. By this criterion, we surmised that eight of the targeted amino acids are important: Phe18 (1.5% as active as wild-type when replaced by alanine); Thr40 (<1%); Asp76 (2.5%); Asn103 (<1%); Asp140 (<1%); Pro142 (7.2%); Tyr148 (12%); and Pro185 (11%). The eight important residues identified presently, and the three defined previously (Asp68, Glu91, Trp143) are highlighted in shaded boxes in Figure 1.

The essentiality of Phe18 might explain the observed loss of methyltransferase activity upon deletion of the segment from aa 15–30 (Figure 2B). Nine residues did not meet the cutoff criterion for functional relevance in the alanine scan: Phe36 (19%); Phe37 (29%); Ser38 (20%); Trp107 (21%); Pro141 (30%); Val146 (87%); Leu184 (37%);
Arg186 (73%); and Asn187 (18%). These nine residues were not subjected to further mutational analysis.

Structure–function relationships and mechanistic insights

We introduced conservative substitutions at nine of the important/essential side chains (all except the two prolines). Eighteen conservative mutants were produced in bacteria and purified by Ni-agarose chromatography (Figure 3B). Their methyltransferase titration profiles are shown in Figure 5. Their specific activities were normalized to the wild-type value (Table 1). Interpretable structure–activity relationships were gleaned at every residue, as discussed below.

Asp68 and Asp76 are located within the Tgs2 counterpart (66VI
   GTACVGGD76) of a canonical AdoMet-binding motif. Asp68 is strictly conserved in other Tgs homologs (Figure 1). We found that whereas the Tgs2 D68A mutant was inactive (<1% of wild-type), introducing a glutamate partially restored function (to 12% of wild-type), whereas asparagine was less beneficial (4.5%). Replacing Asp76 with glutamate revived activity to 33% of wild-type (compared to 2.5% for D76A), whereas asparagine was again less effective (10%). The requirement for a carboxylate at Tgs2 position 76 is notable in light of the fact that an amide functional group (Asn or Gln) is present naturally at the equivalent position in several Tgs2 homologs (Figure 1). The Tgs2 AdoMet-binding motif is similar to that of the microsporidian cap guanine-N7 methyltransferase Ecm1 (68VL
   DLGVGKKGD78), for which a crystal structure is available in the AdoMet-bound state (27). The structure–activity relationships for Tgs2 Asp68 and Asp76 are concordant with those seen at Ecm1 Asp70 and Asp78,
i.e. Ala and Asn are defective in vitro and in vivo, while Glu restores Ecm1 activity (13). Reference to the Ecm1 structure suggests that Tgs2 Asp68 and Asp76 are likely to engage in a network of water-mediated contacts in vitro and in vivo, while a glutamate restores activity (13). We surmise that Tgs2 requires a longer main-chain to carboxylate linker to attain this essential AdoMet contact than does Ecm1.

We find that four of the important residues of Tgs2 map to a conserved peptide (DPWGGVGY148) of the Tgs clade (Figure 1) that was proposed to comprise a binding site for the m7G methyl acceptor. A similar peptide (DFPYGIRESI) is found in the S. cerevisiae Trm11 protein, which is required for the formation of a modified N2-monomethylguanosine nucleoside in yeast tRNA (37). An alanine mutation at Asp291 of yeast Trm11 virtually abolished tRNA guanosine N2-methylation activity in yeast extracts (37). We were intrigued by the prospect that the mutational analysis of this motif in Tgs2 might illuminate the distinctions between Giardia Tgs2 and S. pombe Tgs1 with respect to the ability to add one versus two methyl groups at guanine N2. We focused especially on Asp140, which is a serine in S. pombe Tgs1 and other Tgs1 homologs (Figure 1). Thus, we included a serine change (along with conservative mutations of aspartate to asparagine and glutamate), thinking that D140S might confer on Tgs2 the ability to synthesize a TMG cap in vitro. We found that every substitution for Asp140 abolished Tgs2 methyltransferase activity (Table 1), signifying that Asp140 is stringently required for catalysis by the Giardia enzyme. This finding contrasts with the previous report that TMG cap formation in vivo was unaffected when the corresponding serine of S. cerevisiae Tgs1 (Ser175) was replaced by alanine (24).

Trp143 in the DPWGGYVGY148 motif was also strictly essential for Tgs2 activity, insofar as its replacement with phenylalanine (an alternative aromatic side chain) or leucine (a bulky hydrophobic side chain) phenocopied the severe effects of the alanine change. Trp143 is a good candidate to engage in a cation-π stacking interaction with the m7G substrate. An intriguing prospect is that the tryptophan ring nitrogen might also engage in a critical hydrogen bond, which cannot be achieved by Phe or Leu. Different structure–activity relationships were apparent at the other important aromatic side chain of the DPWGGYVGY148 motif, Tyr148, whereby phenylalanine resulted in a gain of function (to 39%) over Y148A (12%) or Y148L (4.7%). Thus, the aromatic quality of this residue is the key property.

Asn103 is strictly essential for Tgs2 methyltransferase activity; the N103D and N103Q mutants were catalytically defective (<1% activity), just like N103A (Table 1). Asn103 is conserved in all the Tgs homologs (Figure 1). Although there is no atomic structure available for any member of the Tgs family, it has been proposed (24) that they might be structurally similar to a putative...
Methanococcus methyltransferase (Mj082) for which a crystal structure of the apoprotein has been solved (38). Reference to the Mj082 structure shows that its putative equivalent of Tgs2 Asn103 (Asn96 in Mj082) is not situated anywhere near the probable active site; rather it donates two hydrogen bonds from Asn-N6 to the backbone carbonyl atoms of two secondary structure elements that form the predicted AdoMet binding pocket. We suspect that Asn103 plays a similar critical structural role in insuring the fold of Giardia Tgs2, thus accounting for the drastic effects of all Asn103 mutations.

A clear requirement for the hydroxyl group at essential Tgs2 residue Thr40 emerged from the conservative mutational effects, whereby valine (<1% activity) phenocopied the catastrophic effects of the alanine change, while serine restored activity to 41% of the wild-type level (Table 1). We surmise that the Thr40 hydroxyl engages in a critical hydrogen bond, but we have no basis at present to infer whether this is an interaction with substrates or other constituents of the enzyme. The most proximally located of the essential residues, Phe18, appears to function via its aromatic character, because the conservative leucine change mimicked the drastic effects of F18A (Table 1).

The TMG cap is nonessential in S. pombe

Given the ubiquity of TMG caps in eukaryal species, and the essential roles of TMG-capped RNAs in pre-mRNA splicing and other RNA transactions, it was surprising that uncapped 5.8S RNA was recovered exclusively in the supernatant in both strains (Figure 6). We surmise that Tgs1 is the sole enzyme capable of TMG capping in S. pombe (at least as far as snRNAs are concerned) and that the TMG cap structure is not essential in fission yeast. In contrast, TMG synthesis is essential in Drosophila, i.e. mutations in the putative Tgs1 active site caused lethality at the early pupal stage correlated with depletion of TMG-containing RNAs (39).

The m^7G monomethyl cap is essential in S. pombe

The report that m^7G synthesis can be dispensed with C. albicans (6) is counter to expectations regarding the dependence of translation on the cap N7 methyl group. To discern if Candida is exceptional among fungi in this regard, we queried the effects of ablating the cap guanine-N7 methyltransferase (Pcm1) of S. pombe. We inactivated one pcm1^+ allele in a diploid strain (by replacing the coding sequence with a kanMX cassette) and confirmed correct gene targeting by southern blotting and confirmed correct gene targeting by Southern bloting; not shown). The tgs1Δ cells grew as well as the wild-type tgs1^+ sisters at 30°C (not shown). Thus, the Tgs1 protein is nonessential for growth.

Figure 6. Cap guanine-N2 methylation is not essential in S. pombe. Total RNA from tgs1^+ and tgs1Δ strains of S. pombe was subjected to immunoprecipitation with anti-TMG antibody bound to protein A sepharose beads. Aliquots of input RNA (I), immunoprecipitated RNA (P) and unbound RNA from the supernatant (S) were analyzed by northern blotting using probes to detect U1, U2, U4 and U5 snRNA and the uncapped 5.8S RNA as described in methods section. Autoradiograms of the blots are shown.
To explore whether cap methylation activity is essential (as opposed to some other property of the Pcm1 protein), we established a plasmid shuffle assay for complementation of the S. pombe pcm1Δ strain. We introduced into the pcm1Δ pcm1Δ diploid a wild-type pcm1+ gene on a plasmid marked with the S. pombe ura4Δ gene. This maneuver allowed us to recover uracil-prototrophic G418-resistant pcm1Δ haplotypes after sporulation, because the pcm1+ plasmid complements the chromosomal null mutation. Into this strain, we introduced plasmids marked with LEU2 and bearing or lacking a wild-type pcm1+ gene. Leu+ transformants were screened for growth on medium containing FOA. We found that the strain containing the LEU2 pcm1+ plasmid gave rise to FOA-resistant colonies, whereas the strain containing the LEU2 vector failed to do so (Figure 7). With the plasmid complementation assay validated, we proceeded to test whether a heterologous cap guanine-N7 methyltransferase could support S. pombe growth. We found that a LEU2 plasmid bearing the ABD1 gene encoding S. cerevisiae cap methyltransferase was able to support growth under FOA selection (Figure 7). Thus, budding yeast Abd1 can function in lieu of Pcm1. We had shown previously that S. pombe Abd1 can function in growth. We found that a plasmid bearing the gene encoding S. cerevisiae cap methyltransferase was able to support growth under FOA selection (Figure 7). These three Pcm1 mutants were also unable to complement growth of an S. cerevisiae; abd1Δ strain in a plasmid shuffle assay (not shown). We conclude that Pcm1 and its cap guanine-N7 methyltransferase activity are essential for growth of S. pombe. Given that Tgs1 is not essential in S. pombe, we infer that a monomethyl cap is sufficient for the function of the small RNAs that normally have TMG caps in fission yeast.

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**Conflict of interest statement.** None declared.

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