A domain of the actin binding protein Abp140 is the yeast methyltransferase responsible for 3-methylcytidine modification in the tRNA anti-codon loop

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ABSTRACT

The 3-methylcytidine (m^{3}C) modification is widely found in eukaryotic species of tRNA^{Ser}, tRNA^{Thr}, and tRNA^{Arg}; at residue 32 in the anti-codon loop; and at residue e2 in the variable stem of tRNA^{Ser}. Little is known about the function of this modification or about the specificity of the corresponding methyltransferase, since the gene has not been identified. We have used a primer extension assay to screen a battery of methyltransferase candidate knockout strains in the yeast *Saccharomyces cerevisiae*, and find that tRNA$^{Thr(IGU)}$ from *abp140-Δ* strains lacks m^{3}C. Curiously, Abp140p is composed of a poorly conserved N-terminal ORF fused by a programed +1 frameshift in budding yeasts to a C-terminal ORF containing an *S*-adenosylmethionine (SAM) domain that is highly conserved among eukaryotes. We show that *ABP140* is required for m^{3}C modification of substrate tRNAs, since primer extension is similarly affected for all tRNA species expected to have m^{3}C and since quantitative analysis shows explicitly that tRNA$^{Thr(IGU)}$ from an *abp140-Δ* strain lacks m^{3}C. We also show that Abp140p (now named Trm140p) purified after expression in yeast or *Escherichia coli* has m^{3}C methyltransferase activity, which is specific for tRNA$^{Thr(IGU)}$ and not tRNA^{Phe} and occurs specifically at C$_{32}$. We suggest that the C-terminal ORF of Trm140p is necessary and sufficient for activity in vivo and in vitro, based on analysis of constructs deleted for most or all of the N-terminal ORF. We also suggest that m^{3}C has a role in translation, since *trm140-Δ trm1-Δ* strains (also lacking m^{2,2}G$_{26}$) are sensitive to low concentrations of cycloheximide.

Keywords: 3-methycytidine; *S. cerevisiae*; *TRM140*; *ABP140*; tRNA; frameshift

INTRODUCTION

tRNA molecules are widely modified in nature, with a total of 92 chemically distinct tRNA modifications identified in different organisms and entered in the RNA modification database (http://rna-mdb.cas.albany.edu/RNAmods/). In the yeast *Saccharomyces cerevisiae*, 25 different tRNA modifications are found, at 36 different positions, among the 34 sequenced tRNA species (Sprinzl and Vassilenko 2005), with an average of 12.6 modifications per tRNA species (Phizicky and Hopper 2010). These modifications display two broadly defined roles. First, a number of modifications in and around the anti-codon loop play distinct roles in translation, affecting charging of the tRNA with the correct amino acid,

reading frame maintenance, or the efficiency and/or accuracy of translation (Phizicky and Hopper 2010). Second, a number of modifications in the main body of the tRNA remote from the anti-codon have a role in the biogenesis or maintenance of tRNA, and mutations in these modifications lead to turnover of pre-tRNA or mature tRNA by different quality control pathways (Kadaba et al. 2004; Chernyakov et al. 2008). Over the past decade, the enzymes and their cognate genes responsible for most of these modifications have been defined (Phizicky and Hopper 2010).

One of the remaining unidentified yeast genes encoding modification enzymes is the methyltransferase responsible for formation of 3-methylcytidine (m^{3}C). The m^{3}C modification is widely found in eukaryotic tRNA^{Ser}, tRNA^{Thr}, and tRNA^{Arg} tRNA species at residue 32 of the anti-codon loop (Sprinzl and Vassilenko 2005). m^{3}C$_{32}$ is found in all four characterized cytoplasmic tRNA^{Thr} species from animals or fungi, in bovine liver mitochondrial tRNA$^{Thr(UGU)}$, in all but one of the 15 characterized cytoplasmic tRNA^{Ser} species from plants and animals, in four of the five characterized

fungal cytoplasmic tRNA^{Ser} species with an encoded C$_{32}$ residue, and in bovine mitochondrial tRNA$^{Ser(UGA)}$, as well as in two of the five characterized tRNA^{Arg} species from animals. Curiously, m³C is also found at residue e2 of the variable loop of the seven cytoplasmic tRNA^{Ser} species from animals. In the yeast *S. cerevisiae*, m³C is found at C$_{32}$ of tRNA$^{Thr(IGU)}$, tRNA$^{Ser(UGA)}$, and tRNA$^{Ser(CGA)}$ (Weissenbach et al. 1977; Sprinzl and Vassilenko 2005; Dunin-Horkawicz et al. 2006).

It seems likely that the m³C$_{32}$ modification would have an important role in the cell, since residue 32 of the anti-codon loop interacts with residue 38 to maintain the structure of the anti-codon loop (Auffinger and Westhof 1999, 2001), an interaction that is known to be important for decoding accuracy (Olejniczak et al. 2005; Olejniczak and Uhlenbeck 2006; Ledoux et al. 2009). However, since its discovery some 47 yr ago (Hall 1963), little has been discovered about the biology and function of m³C or about the rules that govern the specificity of this modification in vivo.

To begin to define the biochemical basis of m³C modification in the cell and the roles of the modification, we have carried out a systematic screen to identify the gene encoding the m³C methyltransferase responsible for this modification. To our surprise, we find that the methyltransferase is the actin-binding protein Abp140p (Asakura et al. 1998), which is necessary and sufficient for the m³C modification (now renamed *TRM140* to indicate its tRNA modification activity). It is intriguing that *ABP140* is translated by a programed frameshift (Farabaugh et al. 2006b), which fuses an upstream ORF that is variable among different species and implicated in organization of the actin cytoskeleton in the cell (Asakura et al. 1998), with a downstream ORF that, like m³C, is conserved among eukaryotes. We find that this downstream ORF, which has a SAM binding domain (Katz et al. 2003), is necessary and sufficient for m³C methyltransferase activity in vitro and in vivo. We also find that although *abp140-Δ* strains have no obvious growth phenotype, *abp140-Δ trm1-Δ* cells, which are missing both m³C and m^{2,2}G, have a distinct, but modest, growth defect in the presence of a translation inhibitor, suggesting a role for m³C in translation.

RESULTS

The yeast *ABP140* gene is required for formation of m³C

To determine the gene responsible for m³C modification of tRNA in yeast, we first developed a sensitive primer extension assay to detect the modification, using yeast tRNA$^{Thr(IGU)}$, which is known to contain m³C$_{32}$ (Weissenbach et al. 1977). We purified tRNA$^{Thr(IGU)}$ from wild-type yeast cells, annealed a labeled primer designed to pair with the tRNA from residue 55 in the T-loop through residue 35 in the anti-codon loop (Fig. 1A), and extended the primer with reverse

transcriptase. Extension resulted in a product that terminated at residue 33 because of the presence of the m³C modification at C$_{32}$ (Fig. 1B, lanes a–c). In contrast, primer extension of the same tRNA after treatment with the bacterial demethylase AlkB to eliminate the m³C residue (Aas et al. 2003), as well as the m^{2,2}G modification, resulted in a fully extended primer extension product (Fig. 1B, lanes d, e). Thus, the presence and absence of the m³C residue could be scored based on the length of the primer extension product.

To identify the gene responsible for m³C modification of tRNA$^{Thr(IGU)}$, we employed the primer extension assay to
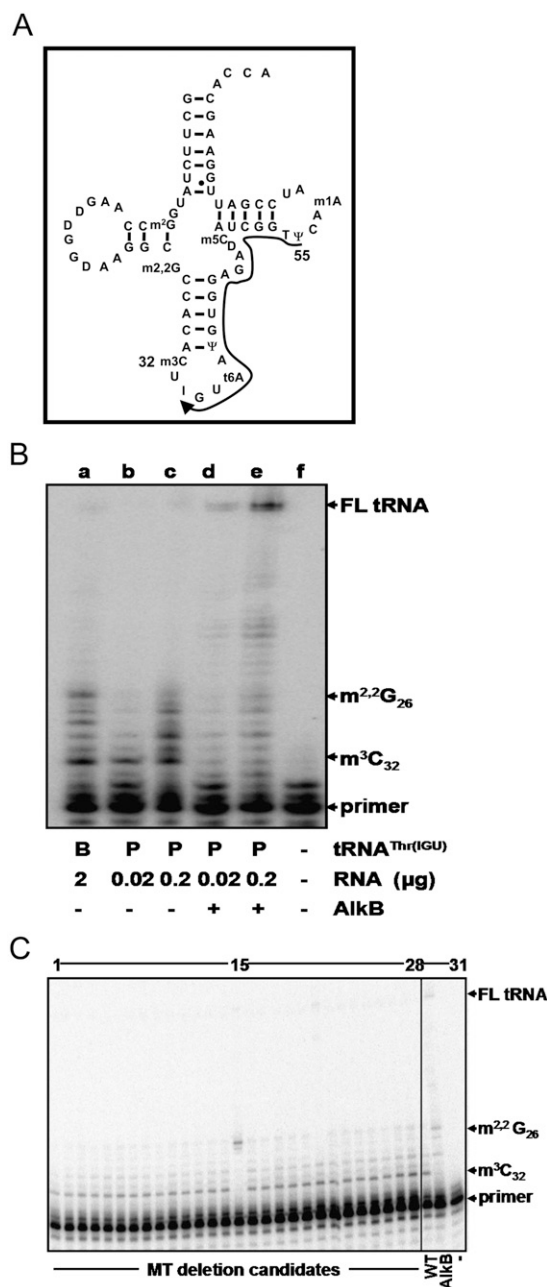

FIGURE 1. (Legend on next page)

screen candidates from the yeast haploid deletion collection. Based on a text search for methyltransferases in SGD (http://www.yeastgenome.org/cgi-bin/search/textSearch.pl?query=methyltransferase&type=headline), a search of Gcd14-like domains in SGD (http://www.yeastgenome.org/cgi-bin/protein/domainPage.pl?dbid=S000003661), and a methyltransferase search in the Modomics database (Dunin-Horkawicz et al. 2006), we came up with a list of 86 strains with deletions of nonessential genes that encode known methyltransferases, uncharacterized ORFs with similarity to known methyltransferases, or known partners of methyltransferase proteins (Supplemental Table S1). RNA prepared from 77 of these strains was analyzed, yielding one candidate with an extended primer extension product (Fig. 1C). This strain has a deletion of ABP140, which encodes an actin binding protein that co-localizes with actin cables and cortical actin patches (Asakura et al. 1998) and which is synthesized as a programed frameshift of ORF YOR239W with YOR240W (Farabaugh et al. 2006b).

To confirm that ABP140 is responsible for the primer extension block indicative of m^{3}C modification of tRNA$^{Thr(IGU)}$, we reconstructed and tested abp140 deletion strains. As anticipated, we find that the primer extension block is absent in strains lacking either one or both ORFs comprising ABP140 (Fig. 2A) and is restored in the deletion strains by introduction of a CEN vector bearing wild-type ABP140, but not by the empty vector (Fig. 2A).

To determine if other tRNA^{Thr} and tRNA^{Ser} species are also substrates for the presumed m^{3}C modification directed by ABP140, we used a similar primer extension assay. Both tRNA$^{Thr(UGU)}$ and tRNA$^{Thr(CGU)}$ from wild-type cells also have the primer extension block expected of m^{3}C modification at residue 32 (Fig. 2B, lanes e, h), but this primer

FIGURE 1. Identification of the gene responsible for m^{3}C modification of yeast tRNA$^{Thr(IGU)}$. (A) Schematic of tRNA$^{Thr(IGU)}$ and primer used for assay of m^{3}C$_{32}$. Modifications found on tRNA$^{Thr(IGU)}$ are indicated (Weissenbach et al. 1977). The oligonucleotide used for primer extension by reverse transcriptase anneals to residues 55–35. Primer extension of tRNA$^{Thr(IGU)}$ species that contain m^{3}C$_{32}$ will terminate at residue 33, whereas species lacking m^{3}C$_{32}$ will terminate at residue 27 before m^{2,2}G$_{26}$. m^{2}G indicates N2-dimethylguanosine; D, dihydrouridine; m^{2,2}G, N$_2$, N$_2$-dimethylguanosine; I, inosine; t^{6}A, N6-threonylcarbamoyladenosine; Ψ, pseudouridine; m^{5}C, 5-methylcytidine; T, ribothymidine; and m^{1}A, 1-methyladenosine. (B) Validation of primer extension assay with AlkB-treated tRNA$^{Thr(IGU)}$. tRNA$^{Thr(IGU)}$ purified from wild-type yeast cells was treated with the bacterial demethylating enzyme AlkB, and the resultant product was analyzed by the primer extension assay, as described in Materials and Methods. B indicates bulk RNA; P, purified tRNA; FL, full-length; a, 2 μg untreated bulk RNA; b, c, untreated tRNA$^{Thr(IGU)}$ (0.02 μg and 0.2 μg); d, e, AlkB-treated tRNA$^{Thr(IGU)}$ (0.02 μg and 0.2 μg); and f, no input RNA. (C) An abp140-Δ strain lacks the m^{3}C primer extension block. Bulk RNA from a set of candidate deletion strains was screened by primer extension. RNA from the abp140-Δ strain lacking ORF YOR239W (lane 15) lacks the m^{3}C primer extension block and has a block corresponding to m^{2,2}G$_{26}$. WT indicates RNA from wild-type control strain; AlkB, AlkB treated tRNA$^{Thr(IGU)}$; and −, no RNA.

extension block is absent in RNA from abp140Δ strains (lanes d, g). Similarly tRNA$^{Ser(CGA)}$, tRNA$^{Ser(UGA)}$, and tRNA$^{Ser(GCU)}$ each have the primer extension block expected from m^{3}C modification of the tRNA in wild-type cells, but the primer extension block is not observed in abp140-Δ cells (Fig. 2C).
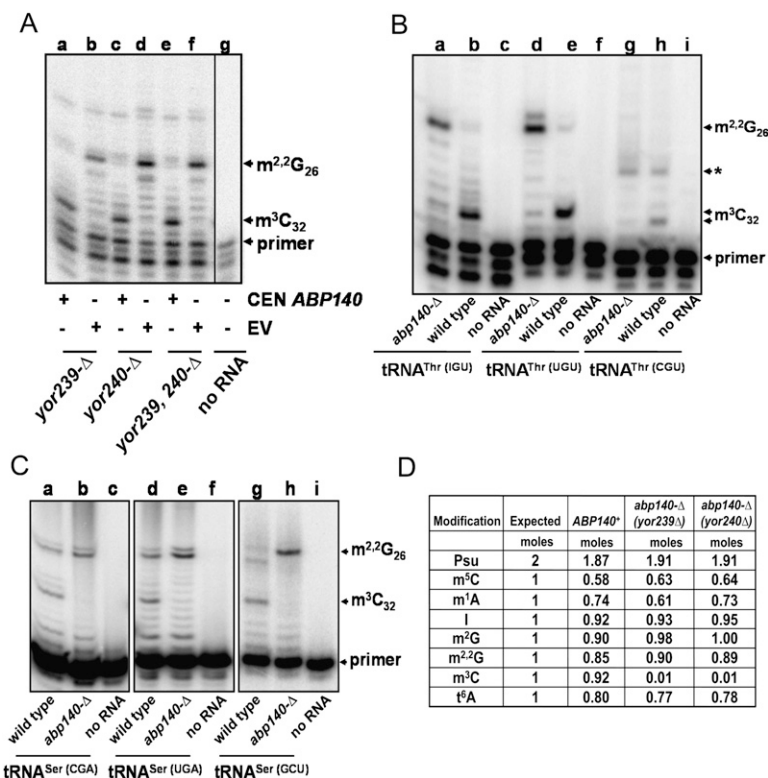
To confirm the interpretation of our primer extension results that the primer extension block at residue 33 is due to m^{3}C modification at residue 32, we directly measured m^{3}C levels in tRNA$^{Thr(IGU)}$. We purified tRNA$^{Thr(IGU)}$ from log phase cultures of the wild-type and deletion strains lacking either YOR239W or YOR240W, digested the RNA to nucleosides, and analyzed the nucleoside content by reverse-phase HPLC chromatography, as described in the Materials and Methods. We find that tRNA$^{Thr(IGU)}$ from wild-type cells has approximately the expected levels of all nucleosides, including 0.92 mol of m^{3}C, whereas tRNA$^{Thr(IGU)}$ from strains lacking either YOR239W or YOR240W has no detectable m^{3}C but has otherwise similar levels of other nucleosides (Fig. 2D). These observations demonstrate directly that ABP140 is required for the m^{3}C modification of tRNA$^{Thr(IGU)}$ in yeast, and therefore we infer that ABP140 is required for m^{3}C$_{32}$ formation for all six tRNA^{Thr} and tRNA^{Ser} species for which m^{3}C is documented.

Based on these results, and the results below, we refer to the gene ABP140 by the name TRM140, in keeping with nomenclature for other tRNA methyltransferases.

Trm140p (Abp140p) is sufficient for m^{3}C modification of tRNA$^{Thr(IGU)}$ in vitro

To determine if Trm140p (Abp140p) is required for m^{3}C modification of tRNA$^{Thr(IGU)}$ in vitro, we prepared crude extracts from the wild-type and trm140-Δ (abp140-Δ) strains and assayed their m^{3}C methyltransferase activity with S-adenosylmethionine (SAM), using tRNA$^{Thr(IGU)}$ transcribed in vitro with [α-^{32}P] CTP. After incubation of the transcripts with extracts, we digested the tRNA with P1 nuclease to generate 5′-[^{32}P]-labeled CMP and modified CMP residues, and resolved the products by thin-layer chromatography (TLC). We find that crude extracts from wild-type yeast cells have m^{3}C methyltransferase activity (Fig. 3A, lanes a–d), since a modified CMP product is detected after resolution of the nucleotide reaction products on a PEI-cellulose TLC plate. This product co-migrates with an authentic labeled m^{3}C spot, made by reaction of [α-^{32}P] CTP-labeled transcript with DMS, followed by P1 nuclease treatment, which is known to generate 5′-[^{32}P]m^{3}C (p★m^{3}C) (Heyman et al. 1994) This p★m^{3}C product is not detected in an extract from the trm140-Δ (abp140-Δ) strain, even after overexposure of the TLC plate (Fig. 3A, lanes e–h), demonstrating that m^{3}C methyltransferase activity in extracts requires Trm140p (Abp140p). Moreover, extracts prepared from cells overexpressing full-length Trm140p with a nucleotide deleted to correct the

FIGURE 2. *ABP140* is required for m^{3}C modification of tRNA^{Thr} and tRNA^{Ser}. (*A*) Lack of the m^{3}C primer extension block is due to lack of *ABP140*. ORFs *YOR239W* and/or *YOR240W* were deleted by transformation of a wild-type strain (BY4741), and 2 μg RNA from these strains was assayed for the m^{3}C primer extension block after transformation with a *CEN ABP140* plasmid (lanes *a,c,e*), or with an empty vector (lanes *b,d,f*), as indicated. (Lane *g*) No RNA. Note that the m^{3}C and m^{2,2}G arrows point to the residue immediately preceding the modified residue that causes the primer extension block. (*B*) *ABP140* is required for m^{3}C modification of other tRNA^{Thr} species. Bulk RNA (2 μg) from a wild-type strain (lanes *b,e,h*) and an *abp140-Δ* strain (*a,d,g*) was assayed by primer extension using 5′-labeled primers designed against tRNA$^{Thr(IGU)}$ (*a–c*), tRNA$^{Thr(UGU)}$ (*d–f*), and tRNA$^{Thr(CGU)}$ (*g–i*). (Lanes *c,f,i*) no RNA controls; *, unexplained primer extension stop or pause in tRNA$^{Thr(CGU)}$. (*C*) *ABP140* is required for m^{3}C modification of tRNA$^{Ser(CGA)}$, tRNA$^{Ser(UGA)}$ and tRNA$^{Ser(GCU)}$. Bulk RNA (2 μg) from a wild-type strain (lanes *a,d*) and an *abp140-Δ* strain (*b,e*) was assayed by primer extension using 5′-labeled primers designed against tRNA$^{Ser(CGA)}$ (*a–c*), tRNA$^{Ser(UGA)}$ (*d–f*), and tRNA$^{Ser(GCU)}$ (*g–i*). (*D*) HPLC analysis of tRNA$^{Thr(IGU)}$ purified from an *abp140-Δ* (*trm140-Δ*) strain. tRNA$^{Thr(IGU)}$ prepared from wild-type and *abp140-Δ* strains was digested to nucleosides and chromatographed on an HPLC column, as described in Materials and Methods.

coli and assayed activity after affinity purification (see Materials and Methods). To maximize production of the full-length protein, we first deleted a nucleotide at the junction between ORF *YOR239W* and ORF *YOR240W* to encode the full-length fusion protein (labeled ff in the figures to indicate frame-fixed), but surprisingly, immunoblot analysis using antibody against the C-terminal tag shows that the expression of the frame-fixed Trm140p in yeast is very similar to that in a parallel construct with the programed frameshift (Supplemental Fig. S1). We find that purified Trm140-ff protein (Supplemental Fig. S2A) exhibits readily detectable m^{3}C methyltransferase activity in vitro (Fig. 3B, lanes a, b), and activity of the protein is very similar whether derived from a frame-fixed construct or the native construct (Supplemental Fig. S3, cf. lanes a–d and e–h), whereas the mock purification control from yeast with an empty vector has no detectable activity (Supplemental Fig. S3, lanes i–m). Activity is more prominent at 0 or 0.1 mM MgCl$_2$ and is significantly reduced at 1 mM MgCl$_2$ and at 10 mM MgCl$_2$ (Supplemental Fig. S3). As is true in yeast crude extracts, Trm140p purified from yeast is specific, since activity is not detected with tRNA^{Phe} (Fig. 3B, lanes k, l).

Both m^{3}C methyltransferase activity and specificity are maintained after expression in *E. coli*, which does not normally have the modification or the activity. The frame-fixed Trm140p purified after expression in *E. coli* (Supplemental Fig. S2B) has readily detect-

need for a programed frameshift yielded about ninefold more m^{3}C methyltransferase activity, based on the titration (Fig. 3A, lanes i–l), demonstrating that Trm140p is the limiting component of methyltransferase activity in extracts. Furthermore, the m^{3}C methyltransferase activity is specific for its tRNA substrate, since no p*m^{3}C spot is detected after assay of [α-^{32}P] CTP-labeled tRNA^{Phe} transcript with either wild-type extracts or extracts from cells overexpressing Trm140p (Fig. 3A, lanes m, o), although tRNA^{Phe} has a C$_{32}$ residue and although the tRNA^{Phe} is clearly able to be modified by another modification activity that is present in the extracts.

To determine if Trm140p is sufficient for m^{3}C methyltransferase activity, we expressed the gene with a C-terminal affinity tag in yeast or an N-terminal affinity tag in *Escherichia*

able m^{3}C methyltransferase activity on tRNA^{Thr} (Fig. 3B, lanes d, e), but not on tRNA^{Phe} (lanes n, o), whereas no activity is detected in the mock purification from the corresponding *E. coli* vector control strain (lanes h, i). This finding demonstrates clearly that Trm140p alone is sufficient for m^{3}C methyltransferase activity and for tRNA specificity in the absence of other yeast proteins, although we have not yet quantitatively compared levels of activity from the protein produced in yeast and *E. coli*.

Trm140p modifies tRNA$^{Thr(IGU)}$ at position 32

Two lines of evidence suggest that Trm140p catalyzes m^{3}C modification at C$_{32}$ of tRNA$^{Thr(IGU)}$, rather than at another
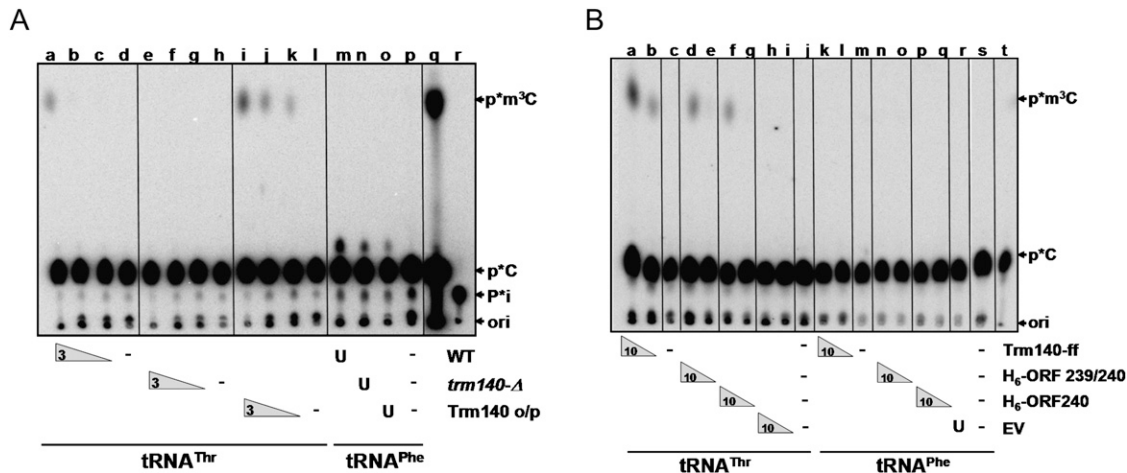
FIGURE 3. Trm140p (Abp140p) is sufficient for m³C methyltransferase activity. (*A*) Trm140p is the limiting component for detection of m³C methyltransferase activity in yeast extracts. tRNA$^{Thr(IGU)}$ transcribed in vitro with [α-³²P] CTP was incubated in methyltransferase buffer containing serial threefold dilutions of extract from wild-type cells (lanes *a–c*), from *trm140-Δ* cells (lanes *e–g*), or from wild-type cells overexpressing frame-fixed Trm140p (Trm140-ff) in which the reading frames of ORFs *YOR239W* and *YOR240W* were deliberately fused (lanes *i–k*) for 2 h at 30°C; then RNA was digested with P1 nuclease; and products were analyzed by chromatography on a PEI-cellulose developed in 0.25 M lithium chloride, as described in Materials and Methods. (*m–o*) Methyltransferase activity assayed with [α-³²P] CTP-transcribed tRNA^{Phe} with the highest concentration of extracts from wild-type cells (*m*), *trm140-Δ* cells (*n*), or wild-type cells overexpressing Trm140p (*o*). (Lane *q*) p*m³C control, generated by DMS-treatment of an [α-³²P] CTP-labeled tRNA$^{Thr(IGU)}$ transcript; (lane *r*) P*i control. (*B*) Trm140p expressed and purified from yeast or from *E. coli* has m³C methyltransferase activity. Methyltransferase activity was examined as described in *A*, with [α-³²P] CTP-transcribed tRNA$^{Thr(IGU)}$, using preparations of frame-fixed Trm140p expressed and purified from yeast (lanes *a,b*), frame-fixed Trm140p expressed and purified from *E. coli* (*d,e*), ORF YOR240W (the C-terminal SAM domain) expressed and purified from *E. coli* (*f,g*), or with equivalent volumes of a parallel mock purification from *E. coli* cells containing an empty vector (*h,i*). *c,j*, no protein; *k–s*, assay of methyltransferase using [α-³²P] CTP-transcribed tRNA^{Phe}; *t*, DMS control.

position. First, formation of m³C requires the presence of the C_{32} residue of tRNA$^{Thr(IGU)}$. Thus, whereas Trm140p readily catalyzes formation of m³C on a wild-type transcript of tRNA$^{Thr(IGU)}$, Trm140p does not have detectable activity on a tRNA$^{Thr(IGU)}$ transcript with a C_{32}A mutation (Fig. 4A, cf. lanes a, b). This result strongly suggests that C_{32} is the target for methylation, although it is possible that the C_{32}A mutation instead alters recognition of the site of modification. Second, primer extension of in vitro modified tRNA$^{Thr(IGU)}$ demonstrates that the modification occurs at residue C_{32}. To this end, we demonstrated that Trm140 activity was not saturated at concentrations of tRNA up to 800 nM, by competition experiments with labeled transcript and purified unlabeled tRNA$^{Thr(IGU)}$ (data not shown). In parallel, we treated 800 nM tRNA$^{Thr(IGU)}$ from a *trm140-Δ* strain with the same amount of Trm140p (or with buffer) in the absence of labeled transcript. The treated products were then subjected to primer extension with the probe for tRNA$^{Thr(IGU)}$. We find that tRNA$^{Thr(IGU)}$ treated with the Trm140p protein generated the m³C primer extension block at residue 33 (Fig. 4B, lane b), while tRNA$^{Thr(IGU)}$ treated only with buffer did not have the m³C primer-extension block (lane c). This result demonstrates that Trm140p modifies C_{32} of tRNA$^{Thr(IGU)}$. Furthermore, since Trm140p modifies only ≤1 mol of cytidine nucleotide per mole of tRNA$^{Thr(IGU)}$, its specificity is limited to residue C_{32}.

ORF *YOR239W* is not required for the methyltransferase activity of Trm140p

Since Trm140p is synthesized by a programed frameshift to fuse ORFs *YOR239W* and *YOR240W*, it seems plausible that both ORFs are required for activity. However, prior experiments suggest that *YOR239W* encodes the protein moiety that interacts with actin (Gao and Bretscher 2008; Riedl et al. 2008), and evolutionary analysis of Abp140p demonstrates that this ORF is poorly conserved, whereas *YOR240W*, which encodes the methyltransferase domain, is widely conserved among eukaryotes (Asakura et al. 1998; Katz et al. 2003; Farabaugh et al. 2006a). Since m³C is also widely conserved among eukaryotes, this analysis suggests that m³C modification activity might be encoded solely by *YOR240W*, although it is also possible that the conserved programed frameshift that fuses the methyltransferase domain and the actin binding domain is catalytically or biologically important within *S. cerevisiae* and related organisms (Farabaugh et al. 2006a).

To address the link between the two domains of Trm140p, we evaluated the m³C methyltransferase activity of the ORF *YOR240W* protein in vitro and in vivo. We cloned ORF *YOR240W* (comprising residues 277-628) with an N-terminal ATG start codon into an *E. coli* expression vector, expressed and purified the protein (Supplemental Fig. S2), and then assayed activity. We find that purified His6-ORF240 has efficient m³C methyltransferase activity
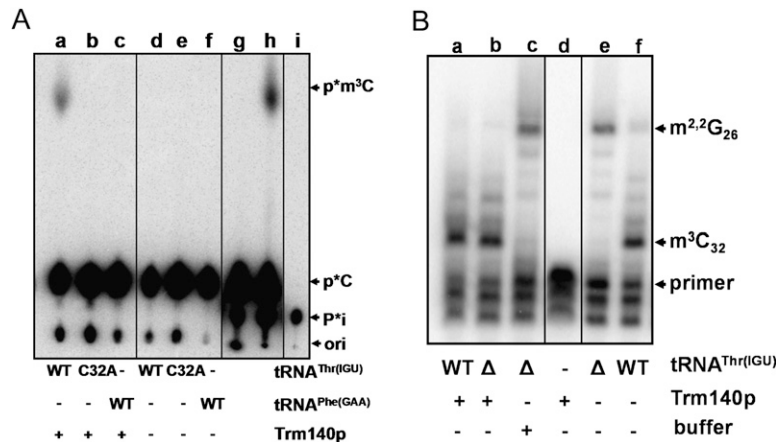
FIGURE 4. Trm140p modifies tRNA^Thr(IGU) at position 32. (*A*) Trm140p does not methylate a C₃₂A variant of tRNA^Thr(IGU). Trm140-ff protein purified from yeast (0.13 μg/μL) was assayed for m³C methyltransferase activity with [α-³²P] CTP-transcribed tRNA^Thr(IGU) (*a*), tRNA^Thr(IGU) C₃₂A variant (*b*), or tRNA^Phe (*c*) for 24 h at 30°C and analyzed as described in Figure 3A. (Lanes *d–f*) tRNAs treated with buffer controls; (lanes *g,h*) untreated (*g*) or DMS-treated (*h*) tRNA^Phe(GAA), used as p*m³C control; (lane *i*) Pi control. (*B*) Modification of unlabeled purified tRNA^Thr(IGU) by Trm140p leads to an m³C₃₂ primer extension block; 0.8 μg tRNA^Thr(IGU) purified from wild-type cells (lane *a*) or trm140-Δ strains (lanes *b,c*) was incubated for 24 h at 30°C in methyltransferase assay buffer with 0.13 μg/μL Trm140-ff protein purified from yeast (lanes *a,b*) or with buffer control (lane *c*), and the location of the m³C modification was analyzed by primer extension, as described in Figure 2. (Lanes *e,f*) primer extension of 0.8 μg untreated tRNA^Thr(IGU) from trm140-Δ (*e*) and wild-type (*f*) strains; (lane *d*) no RNA.

(Fig. 3B, lanes f, g), and that this activity is specific for tRNA^Thr (lanes p, q). Thus, the SAM domain encoded by ORF *YOR240W* (Katz et al. 2003) is sufficient for the m³C methyltransferase activity in vitro, and the N-terminal domain comprising ORF *YOR239W* is dispensable for both activity and specificity in vitro.

To determine if ORF *YOR240W* is sufficient for function in vivo, we deleted the N-terminal domain of *TRM140* comprising ORF *YOR239W* from amino acids 12–276 and expressed *TRM140-Δ12-276* in a *CEN* plasmid. The resulting plasmid (*CEN ORF TRM140-Δ12-276*) was introduced into a *trm140-Δ* strain, and the m³C modification status of tRNA^Thr(IGU) was analyzed by primer extension assay of the RNA. We find that the primer extension block diagnostic of m³C modification is observed when either the *CEN TRM140* plasmid or the *CEN ORF TRM140-Δ12-276* plasmid is present (Fig. 5, lanes a, b), but no primer extension product is observed when the empty vector is expressed (lane c). These results demonstrate in vivo that the m³C methyltransferase activity of Trm140p on tRNA^Thr(IGU) is independent of ORF *YOR239W*, although Trm140-Δ12-276 protein might still bind actin, since the N-terminal 17 amino acids of Trm140p (Abp140p) have been ascribed to this function (Riedl et al. 2008).

trm140-Δ trm1-Δ strains have a mild growth defect in the presence of cycloheximide

Surprisingly, we find that *trm140-Δ* strains have no obvious growth defects under a variety of standard growth

conditions. Thus, when assayed by standard serial dilution spotting experiments, we were unable to detect any growth defect on rich media (YP) or minimal synthetic media (S), with either glucose (D) or glycerol (G) as carbon source, using a temperature range from 18°C–38°C. In addition, the *trm140-Δ* strain is not sensitive to drugs such as cycloheximide, paramomycin, puromycin, and hygromycin (data not shown). The lack of obvious phenotype of a *trm140-Δ* strain is somewhat surprising because of the location of the m³C modification in the anticodon loop at residue 32 (see also Discussion). However, we find that a *trm140-Δ trm1-Δ* strain is mildly sensitive to low concentrations of cycloheximide (0.25–0.3 μg/mL) on rich (YPD) media (Fig. 6A) and that this sensitivity is complemented by reintroduction of a *CEN TRM140* or a *CEN TRM1* plasmid, but not by an empty vector control (Fig. 6B). This mild phenotype in the presence of cycloheximide suggests that lack of the m³C modification does impair translation, albeit mildly.

DISCUSSION

We have provided evidence that the *S. cerevisiae* gene *ABP140* (now called *TRM140*) is necessary and sufficient for m³C modification at C₃₂ of known tRNAs with this
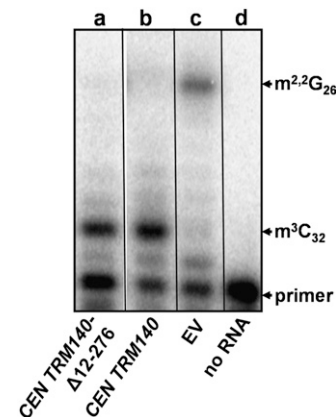
FIGURE 5. ORF *YOR239W* is not required for m³C methyltransferase activity in vivo. A *trm140-Δ* strain was transformed with *CEN* plasmids containing *TRM140-Δ12-276* (lane a), *TRM140* (lane b), or an empty vector (lane c), and bulk RNA (2 μg) prepared from these strains was subjected to primer extension as described in Figure 2. (Lane d) No RNA.
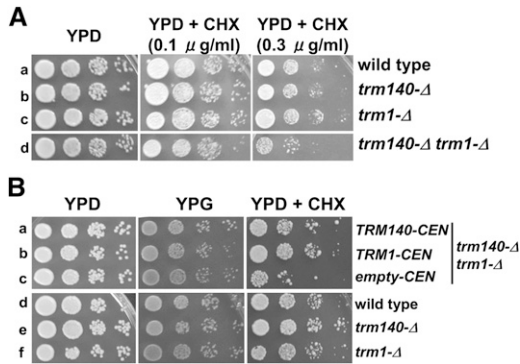
FIGURE 6. A *trm140-Δ trm1Δ* mutant is sensitive to cycloheximide. (*A*) Enhanced cycloheximide sensitivity is observed in a *trm140-Δ trm1-Δ* strain. Wild-type (BY4741), *abp140-Δ* (ySD179), *trm1-Δ* (yLN005), and *abp140-Δ trm1-Δ* (ySD576) strains were grown at 30°C overnight, and 0.3 OD cells were serially diluted by 10-fold, spotted on rich (YPD) medium containing either 0, 0.1, or 0.3 μg/mL cycloheximide, and incubated at 30°C for 2 d (without cycloheximide) or 3 d (with cycloheximide). (*B*) Cycloheximide growth inhibition of a *trm140-Δ trm1Δ* mutant is rescued by expression of *TRM140* or *TRM1*. *trm140-Δ trm1-Δ* cells (ySD576) were transformed with a *LEU2 CEN* plasmid containing *TRM140* (pSD158) or *TRM1* (pMG67A) or with the control empty *CEN LEU2* vector (AVA581), and transformants were grown overnight at 30°C in SD-Leu medium and spotted on YPD, YPG (glycerol) or YPD containing 0.25 μg/mL cycloheximide. Control wild-type (BY4741), *trm140-Δ* (ySD179), and *trm1-Δ* (yLN005) strains were transformed with the same empty vector and tested in parallel. Plates were incubated at 30°C for 2–3 d.

modification. *TRM140* is required for m³C modification, since the primer extension block normally imposed by m³C is absent in *trm140-Δ* mutants in all six tRNA species known to have this modification and since direct examination of nucleosides shows that purified tRNA^Thr(IGU) from a *trm140-Δ* strain specifically lacks m³C that is normally found when the tRNA is purified from wild-type cells. We have also shown directly that Trm140p has m³C methyltransferase activity on tRNA^Thr(IGU) by an assay of protein purified after expression in yeast or *E. coli*. Since this activity is specific for tRNA^Thr compared with tRNA^Phe and occurs specifically at residue 32, we conclude that Trm140p is sufficient for m³C methyltransferase activity, although we have not quantitatively compared the activities, or examined other substrates in vitro.

It is intriguing that Trm140p is synthesized by a programed +1 frameshift in which ORF *YOR239W* is fused to ORF *YOR240W* (Asakura et al. 1998), resulting in the fusion of an actin binding domain with the methyltransferase. Farabaugh and colleagues have done an extensive evolutionary analysis of the *TRM140* (*ABP140*) gene and its programed frameshift, and find that ORF *YOR239W* is poorly conserved, whereas ORF *YOR240W* encodes a highly conserved SAM-methyltransferase domain (Katz et al. 2003; Farabaugh et al. 2006b). Furthermore, they find that the frameshift has been evolutionarily conserved for 150 Myr, since it is extensively found in the budding yeasts, but not in other fungi (Farabaugh et al. 2006b).

There is good evidence that both domains of this protein are functional in yeast but in apparently different biological processes. Although most of the N-terminal ORF *YOR239W* is very poorly conserved, it is clearly implicated in binding to actin in *S. cerevisiae*, since Abp140p was originally identified as an F-actin binding protein that co-localized with cortical actin patches and cytoplasmic actin cables (Asakura et al. 1998), and since the N-terminal 17 amino acids are sufficient for this activity (Riedl et al. 2008). Furthermore, the N-terminal ORF has biological function, since overexpression of the fragment encoding residues 1–218 of *YOR239W* suppresses the lethality conferred by overexpression of a domain of the formin Bni1p (Gao and Bretscher 2008), which nucleates actin filament formation (Pruyne et al. 2002).

Our finding that the C-terminal ORF *YOR240W* is necessary and sufficient for m³C methyltransferase activity in vitro and in vivo is consistent with the high conservation of both the ORF and m³C in fungi, metazoans, and plants and the lack of conservation of either ORF *YOR239W* or the frameshift (Farabaugh et al. 2006b), and suggests that the homologous ORFs encode the m³C-methyltransferase activity. Thus, it appears that Trm140 protein fortuitously combines two ORFs with unrelated function, much like *PUS8/RIB2* encodes the pseudouridyase that modifies position 32 in cytoplasmic tRNAs, as well as the unrelated DRAP (2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate) deaminase that acts in riboflavin biosynthesis (Behm-Ansmant et al. 2004), although it remains to be explicitly determined if actin binding is important in vivo for Trm140p function.

The finding that *trm140-Δ* mutants have no detectable growth phenotype under a variety of conditions is somewhat surprising, given the widespread conservation of the m³C₃₂ modification, the documented importance of the anti-codon loop in translation, the known interaction of residue 32 with residue 38 to affect structure of the anti-codon loop (Auffinger and Westhof 1999, 2001), and the altered ribosome binding and translational fidelity of variants in these residues (Olejniczak et al. 2005; Olejniczak and Uhlenbeck 2006; Ledoux et al. 2009). We note, however, that there are large differences in the severity of growth defects observed in strains with mutations that prevent modification of residues in the anti-codon loop. Thus, severe growth defects are associated with loss of I_{34}, m^1G_{37}, and t^6A_{37} (Gerber and Keller 1999; Bjork et al. 2001; El Yacoubi et al. 2009); less severe but pronounced growth defects are associated with loss of Cm_{32} and Nm_{34}, and Ψ_{38} and Ψ_{39} (Lecointe et al. 1998, 2002; Pintard et al. 2002); and milder but distinct translational differences are associated with loss of $mcm^5s^2U_{34}$, yW_{37}, and i^6A_{37} (Esberg et al. 2006; Waas et al. 2007). The lack of observable phenotype of cells lacking m^3C_{32} is similar to that observed in mutants lacking I_{37} (Gerber et al. 1998), or the pseudouridylase moiety of *PUS8*, which is responsible for

Ψ_{32} modification (Behm-Ansmant et al. 2004). Modification of residue 32 may itself confer only subtle effects on translation, based on the lack of obvious phenotype of *trm140-Δ* mutants and of *pus8* alleles that specifically affect pseudouridylation, although the pronounced growth defect of *trm7-Δ* mutants might be partly due to its modification of residue 32 as well as residue 34 (Pintard et al. 2002).

Indeed, our evidence suggests that m³C modification has a distinct, albeit subtle translation effect, since a *trm140-Δ trm1-Δ* strain is mildly sensitive to low concentrations of cycloheximide, and this sensitivity is complemented by introduction of either a *CEN TRM140* or a *CEN TRM1* plasmid. Since the growth defect is observed in the presence of cycloheximide, which is known to affect the translocation step of translation (McKeehan and Hardesty 1969; Schneider-Poetsch et al. 2010) and requires a strain lacking both m^3C_{32} and $m^{2,2}G_{26}$ (Ellis et al. 1986), it is highly likely that growth is affected due to impaired function of one or more tRNA species during translation. Since these two modifications occur in different regions of the tRNA, one simple interpretation of this result is that the modifications each partially impair function of one or more particular tRNAs in different ways and that the combined result is poor translation by those tRNAs. Alternatively, it is possible that both modifications alter the same property of the target tRNA by each impairing translocation or another step in translation, much like the Hirsh suppressor mutation in the D-stem affects translation fidelity in the anti-codon (Hirsh 1971).

It is tempting to speculate that the programed +1 frameshift has a biological function or is part of a regulatory loop, much as the +1 frameshift required for synthesis of the *S. cerevisiae* Oaz1 is triggered by high concentrations of polyamines in the cell, which then leads to inhibition of ornithine decarboxylase and lower polyamine levels (Palanimurugan et al. 2004), or as the bacterial release factor 2 (RF2) terminates its own synthesis at high concentrations of RF2 but permits +1 frameshifting otherwise (Craigen and Caskey 1986). Although the persistence of the +1 frameshift in *TRM140* for 150 Myr suggests that the frameshift might constitute an important regulatory loop, the fact that the +1 frameshift appears to have been lost within the budding yeast clade by *Saccharomyces castellii* suggests that if the putative regulatory loop is important in budding yeast species, it is not crucial under evolutionary conditions (Farabaugh et al. 2006b). We note that the region just upstream of the frameshift in *TRM140* has an unusual number of threonine codons and serine codons that require decoding by tRNAs with m³C, but we do not yet know their significance.

MATERIALS AND METHODS

Yeast strains

Strains and oligomers used to construct strains are listed in Supplemental Tables S2 and S3. Strains used for genetic tests

and/or analysis of tRNA were derivatives of strain BY4741 (Open Biosystems), and strains used for analysis of biochemical activity were derived from BCY123 (Macbeth et al. 2005), obtained from M.R. Macbeth. The *TRM140* (*ABP140*) gene was deleted in strains BY4741 or BCY123 by PCR amplification of the ble^R cassette of pUG66 (Gueldener et al. 2002), using primers containing sequences directly 5′ and 3′ of *TRM140* (ABP140 − 39 + Phleo and ABP140 + 38 + Phleo), followed by re-amplification with primers ABP140_UP75+Phleo and ABP140_DN75+Phleo, transformation, and selection on YPD media containing 8 μg/mL bleocin. The *trm140-Δ trm1-Δ* strain ySD576 was constructed by PCR amplification of the *trm1-Δ:kanMX* cassette from the corresponding knockout strain (Open Biosystems), using primers TRM1-300 and TRM1 + 366, followed by transformation into ySD179, and selection on YPD containing 300 μg/mL geneticin. Similar approaches were used for construction of other deletion strains, and all deletion strains were confirmed by PCR analysis using flanking chromosomal primers.

Plasmids

Plasmids and oligomers used to construct plasmids are listed in Supplemental Tables S4 and S5. *CEN* plasmids were made by ligation independent cloning (LIC) of appropriate DNA fragments into vectors AVA579 (*CEN URA3*) or AVA581 (*CEN LEU2*), which are derived from conventional *CEN* vectors by incorporation of the LIC site of BG1861 (Alexandrov et al. 2004).

For expression in yeast and affinity purification, *TRM140* was cloned by LIC into vector BG2663 (2μ *URA3* P_{GAL}-ORF-tag), which is similar to BG2483 (Malkowski et al. 2007), and results in fusion of the ORF to a complex tag containing a 3C site, followed by an HA epitope, His6, and the ZZ domain of protein A. This plasmid, pSD142 (2μ *URA3* P_{GAL}-*TRM140*-tag), was used to make pSD242 (2μ *URA3* P_{GAL}-*TRM140*-frame fixed-tag), in which the translational frame of ORF *YOR140W* is fused directly to ORF *YOR239W*, by deletion of nucleotide 829 of the *ABP140* (*TRM140*) coding sequence. This was accomplished using primers 5′ABP140-fix frame and 3′ABP140-fix frame and the Quikchange mutagenesis kit (Stratagene).

For expression in *E. coli* as a His6-3C-ORF, frame fixed *TRM140* from pSD242 (or ORF *YOR240W*) was PCR amplified and cloned by LIC into expression vector AVA421 (Quartley et al. 2009).

Growth and affinity purification of Trm140p from yeast

For purification of Trm140p from yeast, yeast ORF expression plasmids pSD142 (2μ *URA3* P_{GAL}-*TRM140*-tag) and pSD242 (2μ *URA3* P_{GAL}-*TRM140*-frame fixed-tag) were transformed into strain BCY123 (Macbeth et al. 2005). Transformants were grown as described (Quartley et al. 2009), except that cells were harvested 6 h after induction with 3× YP media containing 6% galactose, yielding 5 OD-L of cells.

Crude extracts were made by bead beating, and tagged protein was purified as described (Quartley et al. 2009) by binding to IgG Sepharose, elution of bound protein with GST-3C protease, removal of protease, and dialysis in buffer containing 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 1 mM $MgCl_2$, and 50% glycerol, followed by storage at −20°C.

Growth and affinity purification of His6-Trm140p, His6-ORF YOR240w, and His6-AlkB from *E. coli*

E. coli expression plasmids were transformed into pLys(S)BL21(DE3) cells, and transformants were grown in 25 mL LB medium containing 200 µg/mL ampicillin, at 30°C overnight, diluted 1:200, grown to an OD of 0.6 at 30°C, induced by addition of IPTG to 2 mM, and grown for 3 h at 30°C. Cells from a 1 L growth were harvested and sonicated in 8 mL of buffer containing 50 mM Tris (pH 7.5), 100 mM EDTA, 4 mM MgCl₂, 5 mM DTT, 5% sucrose, 1 M NaCl, and protease inhibitors, and tagged protein was purified from crude extracts by binding to Talon resin, and elution of bound protein with 250 mM imidazole, followed by dialysis in buffer containing 20 mM Tris-Cl (pH 7.5), 200 mM NaCl, 1 mM DTT, and 50% glycerol and storage at −20°C

Isolation of bulk low-molecular-weight RNA

Yeast strains were grown in YPD or minimal media at 30°C to mid-log phase, cells were harvested in 300 OD pellets, and low-molecular-weight RNA was isolated using hot phenol, as described previously (Jackman et al. 2003). For small-scale isolation of bulk low-molecular-weight RNA, yeast cells were grown in 5 mL liquid YPD or minimal media to an OD of 2.5–3.0, and rapid RNA preps were prepared as described (Schmitt et al. 1990).

tRNA purification

tRNA$^{Thr(IGU)}$ was purified from bulk low-molecular-weight yeast RNA using a 5′-biotinylated oligomer, 5′ Bio tRNA^{Thr} (5′ Bio-AAC CGAGAUCUCCACAUUAC-3′; Integrated DNA Technologies) that is complementary to nucleotides 35–55 of tRNA$^{Thr(IGU)}$, essentially as described (Jackman et al. 2003), followed by desalting and concentration using Amicon MLtra-4 10,000 MWCO columns (Millipore).

Primer extension assays

Oligomers used for primer extension are shown in Supplemental Table S6; 30 pmol primers were labeled with 49 pmol [γ-^{32}P]ATP (7000 Ci/mmol MP Biologicals) using T4 polynucleotide kinase (Roche), followed by removal of excess label using a Micro Bio-spin 6 chromatography column (Bio-Rad), gel purification, phenol extraction, and ethanol precipitation. Primer extension assays were done essentially as described (Jackman et al. 2003). In a 5 µL reaction, 1–2 pmol of 5′ end-labeled primers were annealed to 2 µg of bulk RNA or 0.2 µg purified tRNA by heating to 95°C for 5 min and cooling at room temperature for 30–60 min. The annealed reaction was then extended using 0.8 U AMV reverse transcriptase (Promega) in a 10 µL reaction containing 1× AMV RT buffer and 0.11 mM of each dNTP (A, C, G, and T) for 1 h at 37°C. The extension reaction was stopped using RNA load dye (10 µL formamide containing 0.1% bromophenol blue and 0.1% xylene cyanol) and quick frozen in dry ice. The frozen reactions were heated for 5 min to 95°C, cooled on ice, and loaded on a polyacrylamide sequencing gel containing 4 M urea. The gel was dried and exposed on a phosphoimager cassette.

In vitro transcription

Plasmids EMP1568 and EMP1577 (containing tRNA$^{Thr(IGU)}$ and tRNA^{Phe} under control of T7 polymerase) were digested with Bst N1 for 3 h at 60°C and transcribed withT7 RNA polymerase in a 20 µL reaction mixture containing 50 mM Tris (pH 8), 1 mM spermidine, 30 mM MgCl₂, 7.5 mM DTT, 4 mM ATP, 4 mM GTP, 4 mM UTP, and 0.1 mM CTP, 0.1 mg/mL DNA, and 50–100 µCi [α^{32}P]CTP (3000 Ci/mmol from Perkin Elmer) for 1.5 h at 37°C, followed by 10% PAGE, elution of RNA, and resuspension in 10 µL TE (pH 7.5).

m³C methyltransferase assay

m³C methyltransferase activity was assayed in 20 µL reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol (DTT), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM spermidine, 0.5 mM SAM, 5000–10,000 cpm of the corresponding specifically labeled transcript, and a source of m³C methyltransferase activity (crude extract or purified protein). Reactions were incubated for 4 h at 30°C and then stopped by the addition of 90 µL of 0.5 M Tris-HCl (pH 8.0) containing 20 µg carrier tRNA, followed by phenol extraction and ethanol precipitation of the RNA. RNA was resuspended in 5 µL solution containing 30 mM sodium acetate (pH 5.3), 0.2 mM ZnCl₂, and 1 µg/µL of P1 nuclease and incubated for 1 h at 50°C, and digestion products were applied to PEI-cellulose TLC plates (EM Science) and resolved in buffer containing saturated 0.25 M lithium chloride.

HPLC analysis of nucleosides

Purified tRNA^{Thr} (1.25 µg) isolated from yeast was treated with 0.5 µg of P1 nuclease in buffer containing 30 mM sodium acetate (pH 5.2) and 0.2 mM ZnCl₂ for 16 h at 37°C and then with 8 U of calf intestinal alkaline phosphatase (Roche) in 1× alkaline phosphatase reaction buffer for 1 h at 37°C. The resulting nucleosides were resolved by HPLC as described (Jackman et al. 2003).

AlkB demethylation

Purified tRNA^{Thr} (1.25 µg) isolated from yeast was treated with His6-AlkB from *E. coli* B (purified from a clone obtained from B. Sedgwick [Dinglay et al. 2000] using TALON Resin, as described above) in buffer containing 50 mM Hepes KOH (pH 8), 75 µM ferrous ammonium sulfate, 1 mM α-ketoglutarate, 2 mM ascorbate, and 50 µg/mL BSA for 30 min at 37°C and then stopped as described (Trewick et al. 2002).

DMS methylation reaction to generate 5′[^{32}P]m³CMP

150,000 cpm [α^{32}P]CTP labeled transcript was treated with DMS (1:10 dilution in 100% ethanol) in 20 µl reaction mixture containing 50 mM HEPES (pH 7.5) and 1 mM EDTA for 15 min at 70°C in a fume hood. The reaction was stopped with 300 mM sodium acetate (pH 6) and ethanol precipitated, and the pellet was resuspended and digested with 0.5 µg/µL P1 nuclease for 1 h at 50°C.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

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