Plasticity and diversity of tRNA anticodon determinants of substrate recognition by eukaryotic A37 isopentenyltransferases

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ABSTRACT

The *N*⁶-(isopentenyl)adenosine (i⁶A) modification of some tRNAs at position A37 is found in all kingdoms and facilitates codon-specific mRNA decoding, but occurs in different subsets of tRNAs in different species. Here we examine yeasts' tRNA isopentenyltransferases (i.e., dimethylallyltransferase, DMATase, members of the Δ^2 -isopentenylpyrophosphate transferase, IPPT superfamily) encoded by *tit1*⁺ in *Schizosaccharomyces pombe* and MOD5 in *Saccharomyces cerevisiae*, whose homologs are *Escherichia coli* miaA, the human tumor suppressor TRIT1, and the *Caenorhabditis elegans* life-span gene product GRO-1. A major determinant of miaA activity is known to be the single-stranded tRNA sequence, A36A37A38, in a stem-loop. tRNA^{Trp}_{CCA} from either yeast is a Tit1p substrate, but neither is a Mod5p substrate despite the presence of A36A37A38. We show that Tit1p accommodates a broader range of substrates than Mod5p. tRNA^{Trp}_{CCA} is distinct from Mod5p substrates, which we sort into two classes based on the presence of G at position 34 and other elements. A single substitution of C34 to G converts tRNA^{Trp}_{CCA} to a Mod5p substrate in vitro and in vivo, consistent with amino acid contacts to G34 in existing Mod5p-tRNA^{Cys}_{GCA} crystal structures. Mutation of Mod5p in its G34 recognition loop region debilitates it differentially for its G34 (class I) substrates. Multiple alignments reveal that the G34 recognition loop sequence of Mod5p differs significantly from Tit1p, which more resembles human TRIT1 and other DMATases. We show that TRIT1 can also modify tRNA^{Trp}_{CCA} consistent with broad recognition similar to Tit1p. This study illustrates previously unappreciated molecular plasticity and biological diversity of the tRNA-isopentenyltransferase system of eukaryotes.

Keywords: codon; anticodon loop; tRNA modification; tRNA^{Trp}; wobble base; i⁶A; (isopentenyl)adenosine

INTRODUCTION

The greatest diversity of tRNA modifications occurs on nucleotides 34 and 37 in the anticodon loop (ACL), which optimize codon:anticodon fit in the ribosome and promote translational fidelity (Geftter 1969; Vacher et al. 1984; Ericson and Bjork 1991; Jenner et al. 2010). Here we examine yeasts' tRNA isopentenyltransferases (i.e., dimethylallyltransferase, DMATase, members of the Δ^2 -isopentenylpyrophosphate transferase, IPPT superfamily) encoded by *tit1*⁺ in *Schizosaccharomyces pombe* and MOD5 in *Saccharomyces cerevisiae*, whose functional homologs are *Escherichia coli* miaA, human

tumor suppressor TRIT1, and the *Caenorhabditis elegans* life-span gene product GRO-1, which convert A37 to *N*⁶-(isopentenyl)adenosine (i⁶A37) in their target tRNAs (Dihanich et al. 1987; Soderberg and Poulter 2000; Lemieux et al. 2001; Spinola et al. 2005). A major determinant of miaA activity is a single-stranded sequence A36A37A38 in a stem-loop (Motorin et al. 1997; Soderberg and Poulter 2000).

In *E. coli*, miaA creates i⁶A37 that may be further modified, whereas eukaryotic tRNAs contain i⁶A37 without further modification. The i⁶A37 modification occurs on different subsets of tRNAs in different model organisms, largely consistent with the presence or absence of A36A37A38 in the tRNA. In bacteria, i⁶A37 has been found on all tRNAs for codons with U in the first position—Cys, Leu, Phe, Ser, Trp, and Tyr (Bjork 1995; Persson et al. 1994), whereas in eukaryotes i⁶A37 has been found only on a subset of those tRNAs, consistent with their lack of A36A37A38 in tRNAs for

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2628611>.

Leu, Phe, and sometimes Cys. Specifically, yeasts' tRNA^{Phe} and tRNA^{Leu} contain G37 and are therefore not modified with i⁶A37. tRNA^{Cys}_{GCA} carries i⁶A37 in *S. cerevisiae* (Holness and Atfield 1976) but not *S. pombe*, whose tRNA^{Cys}_{GCA} has G37 (Table 1). However, in apparent discord is *S. cerevisiae* tRNA^{Trp}_{CCA}, which contains A36A37A38 but not i⁶A37 (Keith et al. 1971; Juhling et al. 2009; this study). Thus, one cannot confidently predict which tRNAs will be substrates for i⁶A37 modification based only on sequence, at least in yeast and maybe other eukaryotes. Better understanding of the molecular determinants of substrate specificity for DMATases may inform us about the evolution of this ancient activity. In addition, knowing which tRNAs are modified will be important toward deciphering codon-specific effects during translation in different model organisms. Yet, the basis for tRNA^{Trp}_{CCA} discrimination in yeast is unknown, and the specificity of any eukaryotic DMATase has not been reported.

Synthetic RNA minihelices used to examine MiaA in vitro indicate that sequences required for activity reflect a consensus derived from natural tRNA substrates (Motorin et al. 1997; Soderberg and Poulter 2000). In addition to A36A37A38, miaA requires an anticodon stem of at least 5 bp (Motorin et al. 1997; Soderberg and Poulter 2000). MiaA is 316 amino acids in length, very similar to other bacterial DMATases, whereas eukaryotic DMATases contain an additional C-terminal domain of ~100 amino acids that includes a Zn-finger motif. Crystal structures of Mod5p bound to tRNA^{Cys}_{GCA} reveal interaction of the Zn-finger motif with the top of the anticodon stem, somewhat distant from the catalytic site and adjacent region that contact other nucleotides in the ACL (Zhou and Huang 2008). In addition to A37, which is flipped out into a catalytic pocket similar to MiaA-tRNA^{Phe}_{GAA} (Chimnaronk et al. 2009; Seif and Hallberg 2009), the structures also reveal Mod5p interactions with U33 and C35, as well as contacts to the G34 base (Zhou and Huang 2008).

Our interest in specificity arose as we identified tRNA^{Trp}_{CCA} as a substrate of Tit1p in *S. pombe* and confirmed that tRNA^{Trp}_{CCA} is apparently unmodified at N⁶ of A37 in *S. cerevisiae* despite the presence of A36A37A38. Pursuing

the basis of this, our data argued that differential tRNA trafficking in the two yeasts was not responsible. We then performed modification assays in vitro and in vivo for Tit1p and Mod5p and found that discrimination occurs at the level of substrate recognition. Moreover, discrimination by Mod5p can be reversed by changing a single base of tRNA^{Trp}_{CCA}, C34 to G. These observations are consistent with extensive differences in the amino acid sequences of Mod5p and Tit1p in the region of Mod5p that contacts the G34 base of tRNA seen in existing crystal structures. Mutation of Mod5p in this region led to selective debilitation of activity toward its G34-containing natural substrates. Multiple sequence alignments reveal significant diversity in this region of DMATases. Accordingly, the human TRIT1 protein also has broad substrate recognition, more similar to the *S. pombe* enzyme. Thus, insight into the molecular plasticity and biological diversity of the isopentenyltransferase-tRNA substrate system, which affects codon-specific translation, is revealed.

RESULTS

Mutants in *tit1*⁺, formerly known as *sin1*⁺, derived from a genetic screen after NaNO₂-induced mutagenesis (Thuriaux et al. 1976), exhibit loss of codon-specific tRNA-mediated suppression (TMS) by *sup3-e* (and *sup9-e*), which encodes a serine-inserting tRNA^{Ser}_{UCA} (Kohli et al. 1979; Rafalski et al. 1979; Egel et al. 1980; Janner et al. 1980). The multiple previously described *sin1* mutants were mapped genetically but were not sequenced, and the precise mutations remain unknown (Thuriaux et al. 1976; Kohli et al. 1989). Strain yYH1 contains a weak suppressor tRNA^{Ser}_{UCA} allele (Huang et al. 2005, 2006) derived from *sup3-e* that suppresses a UGA nonsense codon in *ade6-704* and the accumulation of red pigment. Due to a partial suppression phenotype, yYH1 can report either gain or loss of TMS (Huang et al. 2005, 2006). Our approach was to target *tit1*⁺ for deletion from yYH1. The resulting *tit1*-Δ strain, designated yNB5, exhibited loss of TMS as reflected by more red pigment than yYH1 (Fig. 1B). Ectopic *tit1*⁺ rescued this phenotype (Fig. 1A,B, yNB5 + *tit1*⁺). Based on mutagenesis of threonine-19 of MiaA (Soderberg and Poulter 2001), which rendered it catalytically debilitated, and multiple sequence alignment, we mutated Tit1p threonine-12 to alanine. Indeed, *tit1-T12A* was inactive for TMS (Fig. 1A,B). Mid-Western blotting using anti-i⁶A antibody detects the physical presence of this modification on tRNA (Benko et al. 2000). This confirmed absence of the i⁶A modification in *tit1*-Δ and *tit1-T12A* tRNAs, while ectopic *tit1*⁺ restored the modification (Fig. 1C,D). The presence of at least two i⁶A-containing bands that migrated with the tRNAs suggests that substrates other than just the suppressor-tRNA^{Ser}_{UCA}, which is a minor tRNA^{Ser} derived from the tRNA^{Ser}_{UCA} gene (Rafalski et al. 1979; Janner et al. 1980), are modified by Tit1p. Western blotting showed that Tit1p-T12A was expressed at a level comparable to Tit1p

TABLE 1. Differences in i⁶A37 among tRNAs in *S. cerevisiae* and *S. pombe*

Anticodon	<i>S. cerevisiae</i>	<i>S. pombe</i>
CysGCA	i ⁶ A37	G37 ^a
SerAGA	i ⁶ A37	i ⁶ A37
SerCGA	i ⁶ A37	i ⁶ A37
SerUGA	i ⁶ A37	i ⁶ A37
TyrGUA	i ⁶ A37	i ⁶ A37
TrpCCA	A37 ^b	i ⁶ A37

^aG is the templated base at this position; G37 modifications may be present.

^bAs reported (Keith et al. 1971; Juhling et al. 2009); N⁶ of A37 appears unmodified based on in vitro modification by Tit1p.

FIGURE 1. Deletion of *tit1*⁺ causes loss of tRNA i^6A and tRNA-mediated suppression. Various strains growing in EMM with non-limiting (A) or limiting (B) adenine, the latter reflecting tRNA-mediated suppression (TMS) activity. yYH1 (WT, *tit1*⁺), the parent strain of yNB5, was transformed with empty vector (row 1); this strain exhibits partial TMS activity (Huang et al. 2005). yNB5 (*tit1*-Δ) was transformed with empty vector, *tit1*⁺, *tit1*-T12A, or MOD5, as indicated for rows 2–5. (C,D) Analysis of *S. pombe* strains in A and B for i^6A in tRNA by mid-Western blotting (Benko et al. 2000). (C) Ethidium bromide-stained total RNA in TBE-urea gel. (D) Blot of C after incubation with anti- i^6A antibody and secondary processing for chemiluminescence (only the tRNA region is shown). (E) The *tit1*⁺ (lane 1), *tit1*-T12A (lane 2), and empty vector (lane 3) transformed strains from above were analyzed by immunoblotting using anti-HA antibody.

(Fig. 1E), providing evidence that *tit1*-T12A produces an inactive enzyme.

Substrate specificity of Tit1p

To set up an in vitro modification assay similar to that used for MiaA (Soderberg and Poulter 2000, 2001), we used purified recombinant Tit1p and 17-nt or 19-nt synthetic oligo-RNAs representing ASLs as minihelix analogs of tRNAs (Soderberg and Poulter 2000, 2001), ¹⁴C-DMAPP (Dihanich et al. 1987) (dimethylallyl pyrophosphate), and 250 nM Tit1p, which was determined to robustly modify one of our best substrate minihelices, SerAGA-19. We examined stem lengths for TyrGUA and SerAGA ASLs as depicted in Figure 2A. Of these, only the ASLs with a predicted stem of 6 bp were efficiently modified by Tit1p (Fig. 2B). Although natural tRNA substrates have only a 5-bp AC stem, these data suggest that the eukaryotic enzyme requires more contact with the upper part of the AC stem than does MiaA (Motorin et al. 1997; Soderberg and Poulter 2000).

To examine specificity for A36A37A38, we assayed ASLs with substitutions as in Ser-G36, Ser-G38, Ser-G37, and

Ser-G36,G38 (Fig. 2C). Position 36 and 37 substitutions greatly diminished activity (Fig. 2C, lanes 2,3). However, Ser-G38 was modified by Tit1p (Fig. 2C, lane 4), unexpected because MiaA exhibits much reduced activity on similar substrates (Soderberg and Poulter 2000). Ser-C38 and Ser-U38 were not modified (Fig. 2C, lanes 6,7).

Upon finding that Tit1p can modify substrates with G38 ASL, we surveyed the *S. pombe* tRNA genome database (Low 2011). Significantly, no tRNAs that contain A36A37G38 were found among the 186 *S. pombe* tRNA sequences. Thus, although Tit1p may be able to modify substrates containing A36A37G38, no such natural tRNA exists. We were unable to determine if Tit1p could modify a tRNA^{Ser}_{UCA}-G38 in vivo because the mutant tRNA^{Ser}_{UCA}-G38 failed to accumulate in *S. pombe* presumably due to rapid decay or counterselection (data not shown).

The C-terminal Zn finger of Tit1p is important for activity in vitro and in vivo

To try to understand the apparent difference between MiaA and Tit1p in ASL stem length, we focused on the eukaryote-specific C-terminal Zn motif. We deleted 55 amino acids from the C terminus of Tit1p to make Tit1p(1-379) and also mutated both cysteines of the C2H2 Zn finger to alanine (Tit1p-ZnAA). We examined these purified Tit1p proteins by in vitro modification of ASL minihelices, in vitro modification of hypomodified tRNAs isolated from *tit1*-Δ cells, and in vivo by TMS. The mutated proteins were

FIGURE 2. Stem length of anticodon stem-loop (ASL) substrates of Tit1p. (A) Predicted structures of ASL oligos representing tRNA^{Ser} and tRNA^{Tyr} of various lengths; A37 is circled. Nucleotide positions 28 and 33 are indicated for SerAGA-17; numbering is the same for all others. For some, an extra closing G-C base pair was added (lowercase). For TyrGUA-19, an A-U base pair was added for comparison to SerAGA-19. (B) In vitro modification of the ASLs in A using recombinant Tit1p and ¹⁴C-DMAPP. (C) SerAGA-19 (lane 1) and its derivative ASLs with the substitutions indicated above lanes 2–7 were assayed as in B.

inactive on all of the ASL minihelices tested (data not shown).

The purified proteins were examined for activity on tRNAs isolated from *tit1-Δ* yNB5 cells (Fig. 3A,B). Wild-type Tit1p produced two bands (Fig. 3A, lane 1) that reflect tRNA^{Ser} and tRNA^{Tyr} based on their sizes of 82 and 74 nt, respectively, and other characteristics described below. Tit1p(1-379) and Tit1-ZnAA were inactive (Fig. 3A, lanes 2,4). Tit1p suffered no loss of activity when mixed with Tit1p(1-379) or Tit1-ZnAA, indicating that the mutated proteins did not contain an inhibitor (Fig. 3A, lanes 3,5).

For in vivo analysis, the proteins were expressed with HA tags. The mutated proteins were inactive for TMS (Fig. 3C) even though they accumulated in *S. pombe* as did Tit1p-HA (Fig. 3D). The cumulative results with these mutants revealed very good agreement of data obtained with ASLs and cellular tRNAs in vitro, and TMS in vivo.

FIGURE 3. The Tit1p extended C terminus including Zn finger is critical for activity in vitro and in vivo. (A) Equal amounts of purified recombinant proteins were tested for in vitro activity using ¹⁴C-DMAPP and total RNA from yNB5 (*tit1-Δ*) as substrate, with protein(s) as indicated above the lanes. (B) The gel in A stained with ethidium bromide. (C) tRNA-mediated suppression assay of the strains transformed with the expression plasmids indicated. (D) Proteins from the yNB5 strains in C transformed with HA-*tit1*⁺ (lane 1), HA-*tit1*(1-379) (lane 2), and HA-*tit1*-ZnAA (lane 3) were examined by immunoblotting using anti-HA antibody.

tRNA^{Trp}_{CCA} is a substrate of Tit1p in vitro

Although tRNA^{Trp}_{CCA}'s from *S. pombe* and *S. cerevisiae* contain A36A37A38, *S. cerevisiae* tRNA^{Trp}_{CCA} was found to contain unmodified A37 (Keith et al. 1971; Juhling et al. 2009). Two 19-nt ASLs representing tRNA^{Trp}_{CCA} from *S. cerevisiae* and *S. pombe* (Fig. 4A), which migrated with different mobilities, were modified by Tit1p (Fig. 4B, lanes 2,3). These ASLs were resynthesized and again migrated with the relative mobilities seen in Figure 4B even before modification (data not shown).

We next performed the in vitro ¹⁴C modification assay on tRNAs isolated from *tit1-Δ* cells before and after antisense oligo-DNA annealing followed by RNase H treatment to identify, by elimination, individual bands on the gel as specific tRNA isoacceptor substrates of Tit1p (Fig. 4C). tRNA from yYH1 (*tit1*⁺) was not a substrate (Fig. 4C, lane 1), as expected if the tRNAs already contain i⁶A37 or another modification to the N⁶ of A37, whereas tRNA from yNB5 (*tit1-Δ*) was modified (Fig. 4C, lane 2). The electrophoresis used for Figure 4C resolved the yNB5 tRNA into three bands of Tit1p products (Fig. 4C, lane 2). In Figure 4C, lanes 3–10, antisense oligo-DNAs spanning the anticodon loop (ACL) of the specific tRNAs targeted for degradation were incubated with the cellular tRNA and subsequently treated with RNase H, then purified prior to addition of ¹⁴C-DMAPP and Tit1p. The *S. pombe* genome harbors seven genes for tRNA^{Ser}_{AGA}, three genes for tRNA^{Ser}_{UGA}, and one gene for tRNA^{Ser}_{CGA}, all of which contained A36A37A38 (Lowe 2011). Targeting tRNA^{Ser}_{AGA} with antisense oligo and RNase H reduced the amount of upper band but not the two lower bands (Fig. 4C, lane 3). Targeting tRNA^{Ser}_{UGA} or tRNA^{Ser}_{CGA} alone had negligible effect on the amount of upper band (Fig. 4C, lanes 4,5). When tRNA^{Ser}_{AGA} and tRNA^{Ser}_{UGA} or tRNA^{Ser}_{CGA} were targeted simultaneously, the upper band was most diminished (Fig. 4C, lanes 6,7). Thus, as expected based on size, the three potential tRNA^{Ser} substrates of Tit1p migrate as the single upper band. The four genes encoding *S. pombe* tRNA^{Tyr} are of identical sequence (Lowe 2011). Targeting tRNA^{Tyr}_{GUA} caused disappearance of only the lowest band (Fig. 4C, lane 8). The three genes encoding *S. pombe* tRNA^{Trp}_{CCA} are of identical sequence (Lowe 2011). Targeting tRNA^{Trp}_{CCA} led to disappearance of the middle band only (Fig. 4C, lane 9). Targeting both tRNA^{Tyr}_{GUA} and tRNA^{Trp}_{CCA} left only the upper band (Fig. 4C, lane 10). These data suggest that tRNA^{Ser}, tRNA^{Tyr}_{GUA}, and tRNA^{Trp}_{CCA} are substrates for Tit1p in vitro and in *S. pombe*, and that they exhibit the relative mobilities indicated to the right of Figure 4C.

tRNA^{Trp}_{CCA} is a substrate of Tit1p in vivo

Indirect evidence, based on altered chromatographic mobility, had suggested that *S. pombe* tRNA^{Trp}_{CCA} was a substrate of *tit1* (then *sin1*), although detection of i⁶A37, which was confirmed for tRNA^{Ser} and tRNA^{Tyr}, was not reported for

FIGURE 4. Identification of tRNA^{Trp}_{CCA} as a substrate of Tit1p. (A) Nineteen-nucleotide ASL oligos representing tRNA^{Trp}_{CCA} from *S. pombe* and *S. cerevisiae* used in B for in vitro modification by Tit1p; SerAGA-19 (lane 1) from *S. pombe* is a control; Trp-19 from *S. pombe* (lane 2) and Trp-19 from *S. cerevisiae* (lane 3). The *S. pombe* and *S. cerevisiae* ASLs Trp-19 run differently on TBE-urea gel (see text). (C) In vitro modification assay after elimination of specific tRNAs by RNase H, to identify *S. pombe* substrates of Tit1p. (Lanes 1,2) In vitro modification by Tit1p of cellular RNA isolated from *yYH1* (*tit1*⁺, lane 1) and *yNB5* (*tit1*-Δ, lane 2). (Lanes 3–10) RNA from *yNB5* was pre-incubated with oligo-DNA(s) complementary to the anticodon loop of the tRNA(s) targeted for elimination indicated above the lanes, followed by RNase H. The RNA was then purified prior to the Tit1p modification assay. (D–H) Positive hybridization in the absence of i⁶A37 (PHA6) assay to identify in vivo substrates of Tit1p in *S. pombe*. (D) The EtBr-stained gel from which the blot hybridizations below it were derived. Lanes 1–4 and 5–8 contain duplicate samples of 5 μg and 10 μg of RNA from *yYH1* (*tit1*⁺) and *yNB5* (*tit1*-Δ) as indicated. The same blot was probed, stripped, and reprobed sequentially with the probes indicated to the right. (E,G) The ³²P-labeled probes were complementary to the anticodon loop (ACL) region of the tRNAs indicated to the right. (F,H) The ³²P-labeled probes were complementary to the TΨC region of the tRNAs indicated to the right. The relative positions of the ACL and TΨC probes are indicated below the blots.

tRNA^{Trp}_{CCA} (Janner et al. 1980). To examine substrates of Tit1p in vivo, we developed a Northern blot assay that we refer to as positive hybridization in the absence of i⁶A37 (PHA6) to detect i⁶A37 hypomodified tRNA. The PHA6 assay works with the expectation that the bulky isopentenyl group on N⁶ of A37 would interfere with stringent hybridization with a ³²P-oligo ACL probe that spanned the 37 position. Hybridization would be greater in the absence of i⁶A37 modification as in *tit1*-Δ cells and less so in *yYH1* cells in which i⁶A37 was present. By this approach, for a Mod5p substrate tRNA we would expect significantly more ³²P-oligo ACL probe signal in *tit1*-Δ than in *tit1*⁺ cells, after calibration to correct for loading using a ³²P-oligo probe complementary to the TΨC loop of the same tRNA. This was readily observed using an ACL ³²P-probe to tRNA^{Ser}_{AGA} on the blot (Fig. 4E) derived from the gel shown in Figure 4D. The membrane was stripped and hybridized with a ³²P-DNA probe complementary to the TΨC loop of tRNA^{Ser}_{AGA}, which showed no difference between *yYH1* and *yNB5* (Fig. 4F). Having validated this assay for a known substrate, we next examined tRNA^{Trp}_{CCA}. The ACL probe hybridized with

tRNA^{Trp}_{CCA} from *yNB5* but not *yYH1* (Fig. 4G). Control hybridization with a TΨC probe revealed comparable hybridization with tRNA^{Trp}_{CCA} from both strains (Fig. 4H), suggesting that tRNA^{Trp}_{CCA} is modified in *yYH1* but not *yNB5* that has no Tit1p, and that lack of i⁶A37 has no significant effect of the tRNA levels.

In an attempt to understand the apparent discrepancy in the substrate activity of tRNA^{Trp}_{CCA} in *S. cerevisiae* and *S. pombe*, we first wanted to confirm (or not) that tRNA^{Trp}_{CCA} is not modified in *S. cerevisiae*. The PHA6 assay did not reveal a significant difference in ACL hybridization to tRNA^{Trp}_{CCA} in MOD5 (ABL8) and *mod5*-Δ cells, similar to the tRNA^{Trp}_{CCA} TΨC probe (Fig. 5A–C), supporting the previous data (Keith et al. 1971; Juhling et al. 2009). We note that this approach cannot rule out the possibility that a small fraction of tRNA^{Trp}_{CCA} may be modified by Mod5p in vivo.

As discussed below, tRNA introns reside between positions 37 and 38, and in such cases splicing is a prerequisite for i⁶A37 modification. We considered this potentially relevant because all of the tRNA^{Trp}_{CCA} genes in *S. cerevisiae* contain introns, whereas none of the tRNA^{Trp}_{CCA} genes in *S. pombe* do (Lowe 2011). To see if tRNAs with introns may be precluded from i⁶A37 modification, we examined *S. cerevisiae* tRNA^{Ser}_{CGA}, whose gene also

contains an intron at the same position, using the PHA6 blot assay. The tRNA^{Ser}_{CGA} ACL probe showed efficient hybridization with tRNA^{Ser}_{CGA} from *mod5*-Δ but not from MOD5 cells (Fig. 5D,E), consistent with tRNA^{Ser}_{CGA} containing i⁶A37 (Etcheverry et al. 1979; Johansson and Bystrom 2005). Furthermore, *S. cerevisiae* tRNA^{Tyr} contains i⁶A37 (Madison and Kung 1967) despite introns in all of its genes (Fig. 5G; Lowe 2011). Thus, it seemed unlikely that the difference in tRNA^{Trp}_{CCA} modification in *S. cerevisiae* and *S. pombe* was due to differential presence/absence of introns in their genes.

We next subjected RNA from *S. cerevisiae* ABL8 (MOD5) and MT8 (*mod5*-Δ) cells to in vitro modification by Tit1p. A single band from ABL8 (MOD5) was produced (Fig. 5G, lane 1) that was absent after *S. cerevisiae* tRNA^{Trp}_{CCA} was targeted by antisense DNA and RNase H (Fig. 5G, lane 2). This provides evidence that *S. cerevisiae* tRNA^{Trp}_{CCA} is unmodified at the N⁶ of A37 in vivo since it is available for isopentenylation by Tit1p. When RNA from *mod5*-Δ cells was subjected to Tit1p, additional bands were observed as expected (Fig. 5G, lane 3). These additional bands were not observed in Figure 5G, lane 2 presumably because they were

FIGURE 5. *S. cerevisiae* tRNA^{Trp} is not a substrate of Mod5p in vitro or in vivo. (A–E) PHA6 assay of *S. cerevisiae* RNAs from MOD5 replete and *mod5-Δ* cells as described in Figure 4 but using probes complementary to the ACL and TΨC regions of the *S. cerevisiae* tRNAs schematically depicted in F and indicated to the right; the same blot was probed, stripped, and reprobed sequentially. (G) In vitro modification by purified recombinant Tit1p, of *S. cerevisiae* tRNAs from *mod5-Δ* cells after elimination of specific tRNAs by RNase H, to identify in vivo substrates of MOD5. In vitro modification using Tit1p of RNA from ABL8 (MOD5) cells after mock preincubation (lane 1) or preincubation with oligo-DNA antisense to the *S. cerevisiae* tRNA^{Trp}, both followed by RNase H treatment and purification prior to the ¹⁴C-DMAPP modification assay (lane 2). (Lane 3) Tit1p-mediated modification of *S. cerevisiae* RNA isolated from MT8 (*mod5-Δ*) after mock preincubation. (Lanes 4–10) RNA from MT8 (*mod5-Δ*) was preincubated with an oligo-DNA(s) antisense to the anticodon loop of the tRNA(s) targeted for elimination indicated above the lanes followed by RNase H and purification, prior to the Tit1p ¹⁴C-DMAPP modification assay. The tRNAs assigned to the bands are summarized to the right. (H) Comparison of Tit1p and Mod5p for in vitro modification of tRNAs from *S. cerevisiae*. (Lanes 1,2) ABL8 (MOD5) tRNA; (lanes 3,4) MT8 (*mod5-Δ*) tRNA modification by Tit1p and Mod5p. The tRNAs assigned to the bands are summarized to the right. (I) Ethidium-stained gel of the assay in H.

already modified in the MOD5 replete cells, ABL8. Examination by antisense DNA and RNase H treatment identified these as tRNA^{Trp}, tRNA^{Cys}, tRNA^{Tyr}, and tRNAs^{Ser} (Fig. 5G, lanes 4–8). The data indicate that the fastest band from *mod5-Δ* cells is a combination of tRNA^{Trp}_{CCA} and tRNA^{Cys}_{GCA}, both at 72 nt in length. *S. cerevisiae* tRNA^{Ser} and tRNA^{Tyr} are 82 and 75 nt, respectively.

Purified Mod5p and Tit1p differ in specificity for tRNA^{Trp}_{CCA}

We used purified recombinant Mod5p for in vitro modification in parallel with Tit1p (Fig. 5H,I). Tit1p but not Mod5p modified the lowest band corresponding to tRNA^{Trp}_{CCA} in RNA from MOD5 replete cells (Fig. 5H, lanes 1,2). Mod5p produced three bands from *mod5-Δ* RNA (Fig. 5H, lane 4), although with preference for the upper two, as compared to Tit1p (Fig. 5H, lane 3), consistent with selective inactivity for

tRNA^{Trp}_{CCA}. The data in Figure 5G,H suggests that Mod5p does not recognize tRNA^{Trp}_{CCA} as a substrate while it does recognize tRNA^{Cys}, tRNA^{Tyr}, and tRNAs^{Ser}.

Inactivity of tRNA^{Trp}_{CCA} for Mod5p can be reversed by a C34G substitution

To gain insight into the determinants of recognition by Mod5p, we compared the ASL regions of its five natural substrates with the tRNA^{Trp}_{CCA} ASL (Fig. 6A). We examined Mod5p activity on the ASL minihelices and their substituted derivatives. Mod5p modified Ser 19 (Fig. 6B, lane 1) but not the ASL derived from *S. cerevisiae* tRNA^{Trp} (Fig. 6B, lane 2, scTrp-19) or *S. pombe* tRNA^{Trp} (spTrp-19) (data not shown). Our approach was to make substitutions in scTrp-19 that would convert it to a Mod5p substrate, guided by Ser 19 as an end point. We first examined the stem. Changing the C29-G41 base pair in scTrp-19 to A-U to match all other Mod5p substrates did not produce activity (Fig. 6B, lane 3), nor did other base-pair changes in the stem (data not shown). Neither did a triple-base-pair substitution at 27-43, 28-42, and 29-41 in scTrp-19 that made it identical to the Ser 19 stem produce activity (Fig. 6B, lane 4). We then focused on the anticodon.

The natural substrates can be arranged into two groups with distinct characteristics. That is, the tRNAs^{Ser}

(Fig. 6A, upper row) all have a G in position 35; an A, C, or U in position 34; and a long variable arm common to type 2 (or class II) tRNAs, whereas the other group members, tRNA^{Tyr}_{GUA} and tRNA^{Cys}_{GCA}, have pyrimidine in position 35, G at 34, and a short variable arm common to type 1 (class I) tRNAs. In contrast, the nonsubstrate tRNA^{Trp}_{CCA} has pyrimidines in both the 34 and 35 positions and a short variable arm. Indeed, a single substitution of scTrp-19 ASL that replaced C34 with G34 activated it as a substrate for Mod5p (Fig. 6C, lane 4). Substituting C34 with A, or C35 with G, did not convert the scTrp-19 ASL to a substrate (Fig. 6C, lanes 3,5). Thus, the scTrp-19 ASL minihelix with a single substitution converting it to the GCA anticodon (normally found on tRNA^{Cys}_{GCA}) was active for modification by Mod5p. This seems significant since the crystal structures of Mod5p-tRNA^{Cys}_{GCA} (Zhou and Huang 2008) show three side chains of Mod5p (H86, K127, K181) in close contact with the N⁶ oxy group of G34, and one side

We compared the ASL minihelices for Tit1p and Mod5p in parallel (Fig. 6D). Since the only Mod5p substrate whose gene encodes A in position 34, tRNA^{Ser}_{AGA}, contains inosine (I) at this position (Zachau et al. 1966; in Johansson and Bystrom 2005), we also examined ASLs with I34. The ASL with I34 was relatively inactive for both proteins (Fig. 6D, lanes 2,7). While the scTrp-19 CAA and CUA ASLs showed little if any substrate activity for Mod5p, these were more active as Tit1p substrates (Fig. 6D). scTrp-19 UCA showed

We next asked if a single C-to-G substitution at position 34 would be sufficient to convert tRNA^{Trp}_{CCA} from an inactive to an active Mod5p substrate in vivo. For this we cloned the wild-type *S. pombe* tRNA^{Trp}_{CCA} gene and a version containing

TABLE 2. Relative activities (0–5) of Mod5p and Tit1p toward different ACL analogs of tRNA^{Trp} and tRNA^{Ser}

	Mod5p activity	Tit1p activity
Sp-Ser AGA	5	5
Sc-Trp CCA	0	5
Sc-Trp ACA	1	1
Sc-Trp ICA	1	1
Sc-Trp GCA	5	5
Sc-Trp CAA	0	5
Sc-Trp CGA	0	3
Sc-Trp UCA	2	4
Sc-Trp CUA	0	5
Sc-Trp mut 2	0	5
Sc-Trp mut 6	1	4
Sc-Ser CGA	5	ND
Sc-Ser UGA	5	ND

Activity of Mod5p toward one of its best substrates. Sp-Ser-AGA-19 is considered as 100% and scored 5; the others are relative to this. Quantification was by Fuji PhosphorImager. Equimolar concentrations of Mod5p and Tit1p were used and their activities toward Sp-Ser AGA-19 compared in the same gel (data not shown).

a single C-to-G substitution at position 34, used them to transform *S. cerevisiae* ABL8 (MOD5) and MT8 (*mod5-Δ*) cells, and performed the PHA6 Northern blot assay (Fig. 6E–I). Six samples were examined, each with 1× and 2× concentrations of RNA, as indicated above the lanes of Figure 6E, the EtBr-stained gel from which the blot for Figure 6F–I was derived. Transformation with the empty vector showed no hybridization with any of the sptRNA^{Trp}_{CCA}-specific probes (Fig. 6F–H, lanes 1–4) as expected, demonstrating specificity of hybridization. sptRNA^{Trp}_{CCA} isolated from MOD5 and *mod5-Δ* showed comparable hybridization with the ACL probe although with slightly more signal in lane 8 than in lane 6 (Fig. 6F, lanes 5–8). In contrast, the sptRNA^{Trp}_{GCA} bearing the C34-to-G34 substitution showed significantly more hybridization with the ACL probe in *mod5-Δ* than in MOD5 (Fig. 6F, lanes 9–12), providing evidence that tRNA^{Trp}_{GCA} was modified by MOD5. The sptRNA^{Trp}_{CCA} TΨC probe detected comparable amounts of sptRNA^{Trp}_{CCA} and sptRNA^{Trp}_{GCA} (Fig. 6H, lanes 5–12). As a further control, we also probed for endogenous sctRNA^{Ser}_{CGA} using a TΨC probe (Fig. 6I). Quantitation with calibration using the sptRNA^{Trp}_{CCA} TΨC probe suggested that ~10%–15% of the sptRNA^{Trp}_{CCA} may be modified by Mod5p; in contrast, ~85% of the sptRNA^{Trp}_{GCA} was modified by Mod5p (data not shown). The data in Figure 6E–I provided evidence that a C34G substitution was sufficient to activate tRNA^{Trp}_{CCA} as a Mod5p substrate in vivo. The data obtained with ACLs in vitro and tRNA substrates in vivo were largely in agreement.

Mutations to the anticodon binding loop of Mod5p alter tRNA substrate preference

As alluded above, the Mod5p side chains of H86, K127, K181, and Y84 are in close contact to the oxy group at

position 6 and N² amine groups of G34 in the Mod5p-tRNA^{Cys}_{GCA} structure (PDBs 3EPH, 3EPJ, 3EPK, and 3EPL) (Zhou and Huang 2008). Sequence alignment (Supplemental Fig. S1) reveals that these are not conserved by Tit1p and furthermore that K127 follows a loop in the Mod5p-tRNA^{Cys}_{GCA} structure (colored white in Supplemental Fig. S2) that helps shape the G34 binding pocket. Notably, this loop is foreshortened by three or more residues in *S. cerevisiae* and related yeasts *Zygosaccharomyces* and *Kluyveromyces*, relative to *S. pombe*, *Schizosaccharomyces japonicus*, and other species (Supplemental Fig. S3), suggesting that it may contribute to differential substrate recognition. We mutated this region of Mod5p and examined activities on RNA isolated from MOD5 and *mod5-Δ* cells (Fig. 7A,B).

The *S. cerevisiae* tRNAs^{Ser} type 2 substrates of Mod5p migrate as one band with the slowest gel mobility due to their large variable arm, while the G34 group, comprised of tRNA^{Tyr}_{GUA} and tRNA^{Cys}_{GCA}, are shorter and each exhibits distinct faster mobilities (Fig. 5G,H). In *S. cerevisiae* there are 16 genes for the tRNAs^{Ser} substrates, eight genes for tRNA^{Tyr}_{GUA}, and four genes for tRNA^{Cys}_{GCA}. Thus, the relative intensities of the three bands observed after Mod5p-mediated in vitro modification should reflect the net effect of their differential abundances and efficiencies as substrates.

Wild-type Mod5p produced three bands comprising both groups of substrates with the pattern observed in Figure 7A, lane 1, in which the middle band is the most intense. In contrast, the mutated Mod5p-loop-K127D protein exhibited selective decrease in the lower two bands, tRNA^{Cys}_{GCA} and tRNA^{Tyr}_{GUA}, relative to the upper tRNAs^{Ser} group band (Fig. 7A, lane 2). The single point mutated proteins Mod5p-K127D and Mod5p-Y84S produced patterns nearly indistinguishable from wild-type Mod5p (Fig. 7A, lanes 1,3,4) and thereby serve as controls for the loop-mutated Mod5p (Fig. 7A, lane 2). Mod5p-K181H consistently showed slight reduction of the two lower bands relative to the upper band (see legend for Fig. 7B; Supplemental Fig. S4). Quantitative scanning of each of the lanes of the gel in Figure 7A is shown in Figure 7B. The same differential patterns were reproducibly observed for the loop-mutated Mod5p and -K181H proteins in multiple experiments including when different preparations of the *mod5-Δ* RNA were used (Supplemental Fig. S3; data not shown). The Mod5p-loop mutated protein also exhibited selective disproportionate decrease in activity toward a Tyr-19-GUA relative to Ser-AGA-19 and the wild-type Mod5p (Fig. 7C). These data support the idea that for the G34 group of Mod5p substrates (G34, Y35, and short variable arm; tRNA^{Cys}_{GCA} and tRNA^{Tyr}_{GUA}), G34 is a determinant of activity. Mutation of the G34 binding loop of Mod5p selectively inhibits activity toward these substrates more than the non-G34 substrates, tRNAs^{Ser}. Although we hoped that point mutations to Mod5p making it more similar to

FIGURE 7. The anticodon binding loop of Mod5p alters tRNA substrate preference. (A) In vitro modification of *S. cerevisiae* *mod5-Δ* RNA by wild-type Mod5p (lane 1) and various mutated Mod5 proteins (lanes 2–5). Mod5p-loop+K127D (lane 2) contains an 8-amino-acid insertion after position 120 as well as the K127D mutation; the Mod5 proteins used in lanes 3–5 are single point mutations as described above the lanes. (B) Quantitative scanning of each lane of the gel shown in A using a Fuji PhosphorImager. For normalization, the total counts observed in tRNAs^{Ser} in Mod5p (wild-type, lane 1) were considered as 100%, and the others were compared accordingly. Note that the black tracing (Mod5p-K127D) for tRNAs^{Ser} is not visible because it was completely masked (overlaid) by the red tracing (Mod5p wild type). (C) In vitro modification of ASL substrates indicated above the lanes by Mod5p wild type and Mod5p-loop+K127D; (upper panel) EtBr staining of gel autoradiogram shown in lower panel. (D) In vitro modification of *S. cerevisiae* MOD5 (ABL8) and *mod5-Δ* (MT8) RNA by purified recombinant human TRIT1.

Tit1p would activate it for recognition of tRNA^{Trp}_{CCA}, this was not observed.

Human TRIT1 appears similar to Tit1p in its anticodon binding loop length and exhibits analogous substrate specificity for tRNA^{Trp}_{CCA}

According to multiple sequence alignment, the loop in Mod5p containing K127 appears to be foreshortened by internal deletions in the Mod5 proteins from *S. cerevisiae* and its close homologs in *Kluyveromyces lactis* and *Zygosaccharomyces rouxii*, relative to *S. pombe* and *S. japonicus* and other species (Supplemental Fig. S3). Similar loop length and lack of conservation of other residues of Tit1p and TRIT1 relative to Mod5p prompted us to examine TRIT1 substrate specificity for tRNA^{Trp}_{CCA}. Figure 7D shows that purified recombinant human TRIT1 can indeed modify tRNA^{Trp}_{CCA}. We conclude that hTRIT1 and Tit1p exhibit an apparently similar substrate recognition that enables tRNA^{Trp}_{CCA} modification, but differ from Mod5p, which is more restricted.

DISCUSSION

The major new finding reported here is that activity of the DMATase, Mod5p, of the model organism, *S. cerevisiae*, is quite sensitive to the base identity of wobble position 34. Specifically, Mod5p does not recognize tRNA^{Trp}_{CCA} as a substrate despite the presence of A36A37A38. A single base substitution that changes tRNA^{Trp}_{CCA} to tRNA^{Trp}_{GCA} activates it for modification by Mod5p, consistent with crystal structure contacts between G34 of tRNA^{Cys}_{GCA} and Mod5p. This is surprising since examination of MiaA substrates would not have predicted this (Motorin et al. 1997; Soderberg and Poulter 2000). As noted in the Introduction, tRNAs that carry i⁶A in prokaryotes include the full set of those that decode all UNN codons, whereas in eukaryotes the subset of tRNAs with i⁶A is more restricted. Several eukaryotic tRNAs that decode UNN codons lack the A36A37A38 motif and would therefore not be expected to be DMATase substrates. However, the work here shows that i⁶A restriction extends to tRNA^{Trp}_{CCA} in *S. cerevisiae* despite the presence of A36A37A38. This restriction can be overcome by substitution of tRNA^{Trp}_{CCA} to tRNA^{Trp}_{GCA}. Why

eukaryotes restrict i⁶A37 to only a subset of tRNAs is unknown.

Molecular plasticity

It was helpful to sort the natural substrates of Mod5p into two groups, the type 2 group composed of tRNAs^{Ser} that contain A, U, or C at position 34, G at position 35, and a large variable arm; and the type 1 group that differs in that its members, tRNA^{Cys}_{GCA} and tRNA^{Tyr}_{GUA}, has G at position 34, pyrimidine at 35, and a small extra arm. The cumulative data suggest that G34 is an important determinant of Mod5p activity for the tRNA^{Cys}_{GCA} and tRNA^{Tyr}_{GUA} of the type 1 group. Consistent with this, tRNA^{Trp}_{CCA}, which resembles the type 1 group with pyrimidine at position 35 and a small variable arm but lacks G at 34 and is inactive as substrate, can be converted to a Mod5p substrate by a single C-to-G substitution at position 34. However, G34 is clearly not required in the natural group 2 substrates, which all have G at position 35 and a large variable arm. The results suggest that Mod5p and other eukaryotic DMATases can recognize determinants in addition to the major one, A36A37A38, in substrate tRNAs that otherwise differ in anticodon sequence and other features. The data suggest molecular plasticity in the recognition of different types of tRNA substrates that differ, among other features, in the sequence at positions 34 and 35 of their anticodons.

Using synthetic ASLs, we found that Tit1p required a longer stem length than was reported for MiaA. The longer length requirement by Tit1p is consistent with the interpretation that the eukaryotic DMATase prefers more structure at the top of the stem because this is where the eukaryote-specific Zn-finger-containing C-terminal region interacts, as seen in the published crystal structure.

A second conclusion is that the homologous DMATases of two distant yeasts overlap in substrate specificity but do not recognize all of the same tRNAs. Specifically, *S. cerevisiae* Mod5p does not modify tRNA^{Trp}_{CCA}, while *S. pombe* Tit1p clearly does. Examination of the existing cocrystal structure of Mod5p-tRNA^{Cys}_{GCA} provides insight into a structural basis for this since Tit1p has not conserved the amino acid side chains in Mod5p that make base-specific contacts to G34. Therefore, this study illustrates previously unappreciated biological diversity of the tRNA-isopentenyltransferase system of eukaryotes. These findings advance understanding of the functional and mechanistic basis of differential tRNA i⁶A37 modification.

Multiple sequence alignment and mutagenesis of the Mod5p G34 binding loop that contains K127 and comprises a region of significant difference from Tit1p and other DMATases suggested that human TRIT1 may exhibit activity for tRNA^{Trp}_{CCA}, which we then demonstrated. The alignment would also suggest that the pathogenic *Candida* species may also exhibit broad specificity, although this remains to be determined.

We have shown that Tit1p catalyzes isopentenylation on tRNAs in vitro and in *S. pombe*. *Tit1-Δ* cells show loss of TMS that is complemented by ectopic Tit1p but not by a catalytic mutant. This reflects importance of i⁶A37 in the function of suppressor-tRNA in codon-specific TMS. This is consistent with anticodon loop modification effects on ASL structure and activity (Agris 2008) as the isopentenyl group appears to limit anticodon loop width and increase stacking with codon:anticodon bases affecting base-pairing and codon-specific translation (Bjork 1995; Agris et al. 2007). The Tit1p constructs with deletion of the extended C terminus or substitution of two cysteines of the Zn finger greatly reduced activity in vitro and for TMS in vivo. The Mod5p Zn finger interacts with the top of the anticodon stem (Zhou and Huang 2008). We suspect that the 15-nt and 17-nt ASLs were not modified by Tit1p because of inefficient interaction with the Zn finger due to their short stem, although other interpretations are possible.

Species-specific tRNA systems

Reliable genome-scale tRNA gene prediction indicates that tRNA gene copy number and corresponding codon usage vary between related species (Lowe 2011), suggesting that dynamics in decoding systems accompanies genome evolution and speciation. Results reported here, that *S. cerevisiae* and *S. pombe* differ in their subsets of i⁶A37-containing tRNAs (Table 1) and that this is in part due to differential recognition of tRNA^{Trp}_{CCA} by their otherwise homologous DMATases, is consistent with the idea that alterations in the dynamics of tRNA-related decoding systems accompany genome evolution and/or speciation.

Codon-specific translation effects of anticodon loop modification

While i⁶A37 is carried by tRNAs^{Ser}_{AGA}, UGA, and CGA, as well as tRNA^{Tyr} in both *S. cerevisiae* and *S. pombe*, each has another i⁶A37-containing tRNA not found in the other yeast. tRNA^{Cys}_{GCA} carries i⁶A37 in *S. cerevisiae* but not in *S. pombe*, in which tRNA^{Cys}_{GCA} contains G37, as summarized in Table 1. Since i⁶A37 affects codon-specific translation, the mRNAs that are sensitive to it may differ in organisms with different subsets of i⁶A37 tRNAs, including perhaps mRNA subsets with differential synonymous codon use bias (Begley et al. 2007). Indeed, in *S. cerevisiae* the anticodon U34 base modification mediated by Trm9p appears to have been “keyed” to the translation of a specific subset of mRNAs with large bias in their use of the cognate codons (Begley et al. 2007). Somewhat similar perhaps are the effects of the N⁶-threonylcarbamoyl adenosine at position 37 (t⁶A37) of tRNAs decoding ANN codons (El Yacoubi et al. 2009, 2011; Srinivasan et al. 2011). Phenotypes attributable to lack of this modification are pleiotropic (El Yacoubi et al. 2011; Srinivasan et al. 2011), presumably related at least in part to

mRNA-specific effects. Remarkably, deficiency of t⁶A37 promotes de-repression of specific mRNAs that bear upstream ORFs whose translation requires t⁶A37-modified tRNA (Daugeron et al. 2011). Determining whether or not defects in codon-specific translation of different subsets of i⁶A37-hypersensitive mRNAs can account for the phenotypic differences observed for *S. pombe tit1-Δ* mutants and *S. cerevisiae mod5-Δ* mutants will be a challenge for the future.

MATERIALS AND METHODS

RNA and DNA oligos

RNA and DNA oligos were purchased from IDT (Integrated DNA Technologies).

Strains

yYH1 is *h⁻ leu1-32:: [tRNA^{Ser}7T-leu1⁺] ura4-D ade6-704*. yNB5 is *h⁻ leu1-32:: [tRNA^{Ser}7T-leu1⁺] ura4-D tit1Δ-Kan⁺ ade6-704*. yAS99 is *h⁻ ade6-704, ura4-D, leu1-32*. ABL8 and MT8 were obtained from Anita Hopper (Gillman et al. 1991). The medium used for *S. pombe* was YES (3% glucose, 0.5% yeast extract + 225 mg/L adenine, histidine, leucine, uracil, and lysine). Edinburgh Minimal Media (EMM) lacking leucine and uracil was used with adenine at 10 mg/L for tRNA-mediated suppression (TMS) as previously described (Huang et al. 2005, 2006).

Plasmids

tit1⁺ including 600 bp of upstream DNA was cloned into pREP42X from which the NMT promoter was excised so that Tit1p was expressed from its own promoter with an HA tag on the C terminus. Transformed cells were grown in EMM lacking uracil to an O.D.₆₀₀ of 0.5. Mutagenesis was carried out by QuickChange XL (Stratagene). All constructs were verified by sequencing.

S. pombe tRNA^{Trp} was cloned into pRS316. Transformed *S. cerevisiae* cells were grown in SC medium lacking uracil, to an O.D.₆₀₀ of 0.6.

Northern blotting including PHA6 assay

S. pombe cells were grown to an O.D.₆₀₀ of 0.5 in liquid media. RNA was extracted with hot acidic phenol:chloroform. Five micrograms of total RNA was electrophoresed in Novex 10% TBE-urea polyacrylamide gels and transferred to GeneScreen Nylon membranes using Invitrogen iBlot transfer apparatus, cross-linked by UV, and baked under vacuum. Blots were incubated overnight at highly stringent hybridization temperatures with ³²P-labeled oligo-DNA complementary to the RNA species indicated.

Mid-Western blotting

Total RNA was electrophoresed, transferred to nitrocellulose, and processed as above. The membrane was blocked for 1 h at room temperature in 5% skim milk/1× PBS/0.05% Tween 20. Anti-i⁶A antibody (provided by Anita Hopper) was added at 1/50 dilution. After washing three times for 5 min each in 1× PBS/0.05% Tween 20, a secondary anti-rabbit HRP antibody was incubated for 1 h in 1% skim milk/1× PBS/0.05% Tween 20 at 1/500 dilution and

then processed for chemiluminescence. The membrane was washed three times for 5 min each with 1× PBS/0.05% Tween 20 and exposed to a PhosphorImager screen.

Protein extraction and immunoblotting

Cells were grown to an O.D.₆₀₀ of 0.5. Protein was extracted in 150 mM NaCl, 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.5% NP-40, and 0.1 mM PMSF (Phenyl Methylsulfonyl Fluoride) and added fresh, using glass beads and a BioSpec Products MiniBead Beater. Debris was pelleted, and the supernatant was adjusted to 10% glycerol and frozen at −80°C. Protein was analyzed on 4%–12% Bis-Tris PAGE gels (Novex) and stained with Simply Blue SafeStain (Invitrogen). To examine tagged proteins, the gel contents were transferred to PVDF membrane using iBlot apparatus (Invitrogen). Anti-HA antibody was used followed by secondary Ab, and the membrane was processed for chemiluminescence according to standard methods.

Protein purification

DNAs encoding Tit1p and its derivatives Mod5p and TRIT1 were cloned into pET15b (Invitrogen) and expressed in *E. coli* BL21(DE3)pLysS (Invitrogen), each with a His6 tag. Induced protein was purified by Talon metal affinity (Clontech) and the yield quantified by Bradford assay (Bio-Rad).

In vitro tRNA isopentenylolation assay

Twenty micrograms of total RNA purified from yNB5 (*tit1-Δ*) or yYH1 (wild-type) or synthetic ASL at 5 μM final concentration was resuspended in 100 μL of reaction buffer: 50 mM Tris-Cl (pH 7.5), 5 mM MgCl₂, 0.1 mM 2-mercaptoethanol, 1 nmol of ¹⁴C-DMAPP (10 μM, dimethylallyl pyrophosphate), and 100 U of Superasein (Ambion AM2694). Recombinant Tit1p at 250 nM was used for each reaction. After incubation, RNA was extracted with acidic phenol–chloroform. The organic phase was re-extracted with 250 μL of TES (10 mM Tris-Cl at pH 7.5, 10 mM EDTA, 0.5% SDS). The aqueous phase (RNA) was precipitated and resuspended in formamide RNA loading buffer, dried, and electrophoresed on a 12% TBE/urea gel. After drying the gel was exposed to a PhosphorImager screen. For RNase H experiments, a 25-mer antisense oligo DNA complementary to positions 23 to 47 of the tRNA was used.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

We thank V. Cherkasova for insight, assistance, and comments; M. Bayfield, D. Hatfield, M. Ibba, and D. Söll for critical comments; Amanda Crawford for reagents and assistance; and Anita Hopper (Ohio State University, Columbus) for anti-i⁶A antibody and the MOD5 and *mod5-Δ* strains. This work was supported by the Intramural Research Program of the NICHD, NIH.

Received January 13, 2011; accepted July 24, 2011.

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RNA 2011 17: 1846-1857 originally published online August 26, 2011

Access the most recent version at doi:[10.1261/rna.2628611](https://doi.org/10.1261/rna.2628611)

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