Downloaded from www.rnajournal.org on February 14, 2006

RNA

A conditional lethal yeast phosphotransferase (tpt1) mutant accumulates tRNAs with a 2'-phosphate and an undermodified base at the splice junction

S. L. Spinelli, S. A. Consaul and E. M. Phizicky

RNA 1997 3: 1388-1400

References
Article cited in:
http://www.rnajournal.org/cgi/content/abstract/3/12/1388#otherarticles

Email alerting service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or **click here**

Notes

To subscribe to *RNA* go to:
http://www.rnajournal.org/subscriptions/

© 1997 RNA Society

Downloaded from www.rnajournal.org on February 14, 2006

RNA (1997), 3:1388–1400. Cambridge University Press. Printed in the USA.
Copyright © 1997 RNA Society.

A conditional lethal yeast phosphotransferase (*tpt1*) mutant accumulates tRNAs with a 2′-phosphate and an undermodified base at the splice junction

SHERRY L. SPINELLI, SANDRA A. CONSAUL, and ERIC M. PHIZICKY
Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642, USA

ABSTRACT

tRNA splicing is essential in yeast and humans and presumably all eukaryotes. The first two steps of yeast tRNA splicing, excision of the intron by endonuclease and joining of the exons by tRNA ligase, leave a splice junction bearing a 2′-phosphate. Biochemical analysis suggests that removal of this phosphate in yeast is catalyzed by a highly specific 2′-phosphotransferase that transfers the phosphate to NAD to form ADP-ribose 1″-2″ cyclic phosphate. 2′-Phosphotransferase catalytic activity is encoded by a single essential gene, *TPT1*, in the yeast *Saccharomyces cerevisiae*. We show here that Tpt1 protein is responsible for the dephosphorylation step of tRNA splicing in vivo because, during nonpermissive growth, conditional lethal *tpt1* mutants accumulate 2′-phosphorylated tRNAs from eight different tRNA species that are known to be spliced. We show also that several of these tRNAs are undermodified at the splice junction residue, which is always located at the hypermodified position one base 3′ of the anticodon. This result is consistent with previous results indicating that modification of the hypermodified position occurs after intron excision in the tRNA processing pathway, and implies that modification normally follows the dephosphorylation step of tRNA splicing in vivo.

Keywords: intron; in vivo; modification; processing; RNA; splicing; *S. cerevisiae*

INTRODUCTION

tRNA splicing is one of several processing steps in the maturation pathway of yeast and other eukaryotic tRNAs (Westaway & Abelson, 1995). All tRNA transcripts in the yeast *Saccharomyces cerevisiae*, as well as in other eukaryotes, are processed by cleavage of the 5′ leader by RNase P (Lee et al., 1991), trimming of the 3′ trailer by nucleases (Yoo & Wolin, 1997), addition of CCA to the 3′ end by terminal nucleotide transferase (Aebi et al., 1990), and by modification of a number of residues in each individual tRNA (Hopper & Martin, 1992). A subset of tRNA gene families also have introns that are removed by a specialized set of tRNA splicing enzymes. tRNA splicing is essential in both *S. cerevisiae* and humans, because each organism has one or more tRNA gene families, the members of which all have introns (van Tol & Beier, 1988; Hopper & Martin, 1992).

Microinjection of *Xenopus* oocytes with the yeast $tRNA^{TYR}$ gene indicates that tRNA processing and modification are temporally ordered, and that the last steps of the pathway are splicing and modification at positions 34 and 37 in the anticodon loop (Melton et al., 1980; Nishikura & De Robertis, 1981). Although the temporal order of splicing is ambiguous in yeast (O'Connor & Peebles, 1991), there is some evidence for order in the pathway. *rna1*^{ts} mutants accumulate unspliced pre-tRNAs at high temperature (Hopper & Banks, 1978) that are fully mature at their 5′ and 3′ ends and, in the 3 tRNAs examined, specifically lack several modifications: modification of the hypermodified residue at position 37 adjacent to the anticodon; 2′-O-methylation, which occurs mostly in the anticodon loop and and the D loop in these tRNAs; and m^2G, which occurs in two tRNAs at position 10 (Knapp et al., 1978; Etcheverry et al., 1979).

Reprint requests to: Eric M. Phizicky, Department of Biochemistry and Biophysics, Box 712, University of Rochester School of Medicine, 601 Elmwood Avenue, Rochester, New York 14642, USA; e-mail: ephi@bphvax.biophysics.rochester.edu.

Downloaded from www.rnajournal.org on February 14, 2006

A defining characteristic of eukaryotic tRNA splicing is the conserved position of the intron one base 3′ of the anticodon, at position 37 (Hopper & Martin, 1992). The presence of the intron results in a pre-tRNA with a secondary structure similar to mature tRNA, except that the anticodon stem is elongated (Swerdlow & Guthrie, 1984; Lee & Knapp, 1985). In the first step of splicing in both yeast and *Xenopus*, endonuclease binds pre-tRNA (Baldi et al., 1986), measures the length of the anticodon stem to determine the cleavage sites (Mattoccia et al., 1988; Reyes & Abelson, 1988), and excises the intron if it has appropriate structure at its 3′ end (Szekely et al., 1988; Baldi et al., 1992). Endonucleolytic cleavage yields tRNA half-molecules with a 2′-3′ cyclic phosphate and a 5′-OH (Peebles et al., 1983; Attardi et al., 1985). In yeast, cleavage occurs in random order and likely with different subunit catalysts (Miao & Abelson, 1993; Trotta et al., 1997). Each of the four subunits of the yeast endonuclease is essential (Trotta et al., 1997), and a specific endonuclease mutant is defective in pre-tRNA cleavage at the 5′ splice site both in vivo and in vitro (Ho et al., 1990).

The steps following excision of the intron are chemically complicated. In yeast, the half molecules are fused by tRNA ligase in a multistep reaction that leaves a mature tRNA with a splice junction 2′-phosphate (Fig. 1). The 3′ half molecule is phosphorylated by a kinase activity in the presence of GTP, the 2′-3′ cyclic phosphate of the 5′ half-molecule is opened to a 2′-phosphate by a cyclic phosphodiesterase activity, and the two ends are joined by the ligase activity, using ATP to generate the activated 3′ half-molecule donor (Greer et al., 1983; Phizicky et al., 1986; Belford et al., 1993; Westaway et al., 1993). Yeast tRNA ligase (the *RLG1* gene product) acts in vivo to join half molecules because Rlg1 is an essential protein, and conditional

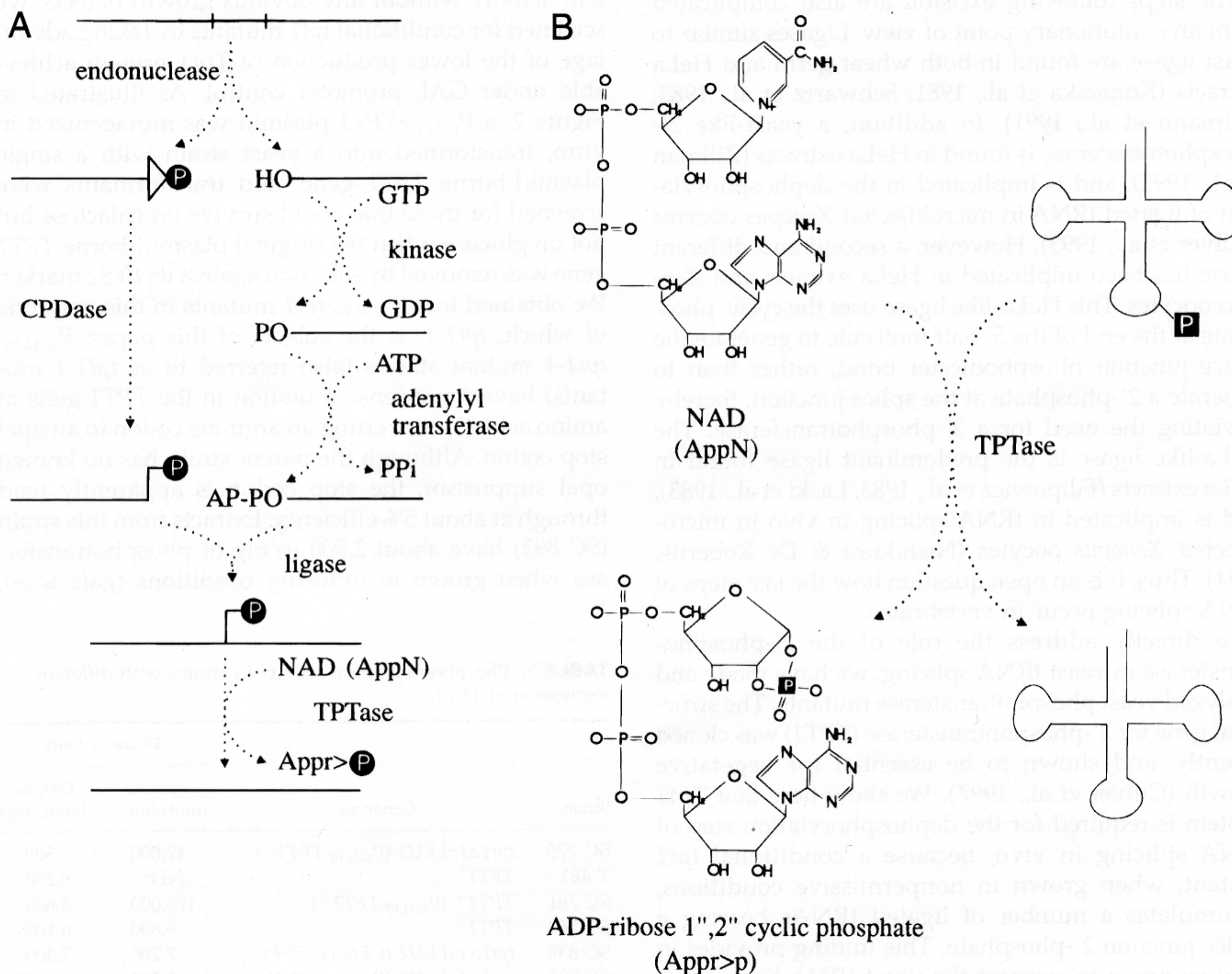

FIGURE 1. tRNA splicing in yeast. **A:** Schematic of the tRNA splicing pathway. Endonuclease is encoded by four genes (Sen2, Sen15, Sen34, and Sen 54) (Trotta et al., 1997); CPDase, kinase, adenylyl transferase, and ligase activities are encoded by a single gene (*RLG1*), and TPTase is encoded by a single gene (*TPT1*). CPDase, cyclic phosphodiesterase; TPTase, 2′-phosphotransferase. **B:** Removal of the splice junction 2′-phosphate. TPTase transfers the splice junction 2′-phosphate from ligated tRNA to NAD to produce ADP-ribose 1″-2″ cyclic phosphate.

Downloaded from www.rnajournal.org on February 14, 2006

rlg1 mutants accumulate unligated tRNA half-molecules (Phizicky et al., 1992). Because removal of the 2′-phosphate precludes ligase function in vitro (Knapp et al., 1979; Westaway et al., 1993), it is widely assumed that ligated tRNA bears an internal 2′-phosphate at the splice junction.

Biochemical evidence suggests that removal of the 2′-phosphate from the splice junction in yeast is catalyzed by a 2′-phosphotransferase that transfers the 2′-phosphate to NAD to form ADP-ribose 1′′-2′′ cyclic phosphate (Appr>p) (McCraith & Phizicky, 1990, 1991; Zillman et al., 1992; Culver et al., 1993). Two lines of evidence support the claim that the 2′-phosphate is removed by this 2′-phosphotransferase. First, only one dephosphorylation activity could be detected in extracts or after chromatography, using a highly sensitive assay for 2′-phosphate removal. Second, the enzyme is highly specific for RNA oligonucleotides with an internal 2′-phosphate, when compared with RNAs with terminal 5′, 3′, or 2′ phosphates.

The steps following excision are also complicated from an evolutionary point of view. Ligases similar to yeast ligase are found in both wheat germ and HeLa extracts (Konarska et al., 1981; Schwartz et al., 1983; Zillmann et al., 1991). In addition, a yeast-like 2′-phosphotransferase is found in HeLa extracts (Zillman et al., 1992), and is implicated in the dephosphorylation of ligated tRNA in microinjected *Xenopus* oocytes (Culver et al., 1993). However, a second and different ligase has been implicated in HeLa extracts and *Xenopus* oocytes. This HeLa-like ligase uses the cyclic phosphate at the end of the 5′ half molecule to generate the splice junction phosphodiester bond, rather than to generate a 2′-phosphate at the splice junction, thereby obviating the need for a 2′-phosphotransferase. The HeLa-like ligase is the predominant ligase found in HeLa extracts (Filipowicz et al., 1983; Laski et al., 1983), and is implicated in tRNA splicing in vivo in microinjected *Xenopus* oocytes (Nishikura & De Robertis, 1981). Thus, it is an open question how the last steps of tRNA splicing occur in vertebrates.

To directly address the role of the 2′-phosphotransferase in yeast tRNA splicing, we have made and analyzed yeast phosphotransferase mutants. The structural gene for 2′-phosphotransferase (*TPT1*) was cloned recently, and shown to be essential for vegetative growth (Culver et al., 1997). We show here that Tpt1 protein is required for the dephosphorylation step of tRNA splicing in vivo, because a conditional *tpt1* mutant, when grown in nonpermissive conditions, accumulates a number of ligated tRNAs bearing a splice junction 2′-phosphate. This finding provides in vivo evidence to support the yeast tRNA ligase joining mechanism. Several of the tRNAs examined from this mutant are also missing the modification at the splice junction residue at position 37, although other modifications appear normal. This finding further defines the temporal order of some of the modification steps.

RESULTS

Isolation of conditional *tpt1* mutants

To study the consequences of a lack of Tpt1p, we sought a conditional mutant strain. Although the *TPT1* gene is essential, we were unable to obtain a conditional lethal phenotype by the usual method of constructing a strain in which the only *TPT1* gene was under control of the tightly regulated *GAL10* promoter. A strain of this genotype [*tpt1-Δ1::LEU2* (*CEN URA3* P_{GAL10}-*TPT1*)] was not detectably compromised for growth when grown in repressing conditions (glucose), although, under these conditions, Tpt1 protein activity levels are 12-fold lower than normally observed in wild-type cells (see Table 1).

Because yeast strains can tolerate so little Tpt1 protein activity without any obvious growth defects, we screened for conditional *tpt1* mutants by taking advantage of the lower production of Tpt1 protein achievable under GAL promoter control. As illustrated in Figure 2, a P_{GAL10}-*TPT1* plasmid was mutagenized in vitro, transformed into a yeast strain with a single plasmid-borne *TPT1* gene, and transformants were screened for those that could survive on galactose but not on glucose when the original plasmid-borne *TPT1* gene was removed by selection against its *LYS2* marker. We obtained four P_{GAL10}-*tpt1* mutants in this way, one of which, *tpt1-1*, is the subject of this paper. P_{GAL10}-*tpt1-1* mutant strains (also referred to as *tpt1-1* mutants) have a nonsense mutation in the *TPT1* gene at amino acid 23, converting an arginine codon to an opal stop codon. Although the parent strain has no known opal suppressor, the stop codon is apparently read through at about 5% efficiency. Extracts from this strain (SC 893) have about 2,500 u/mg of phosphotransferase when grown in inducing conditions (galactose),

TABLE 1. Phosphotransferase activity in strains with different expression of *TPT1*.

		TPTase activity	
Strain	Genotype	Galactose (units/mg)	Glucose (units/mg)
SC 775	*tpt1Δ1::LEU2* (P_{GAL10}-$TPT1^+$)	42,000	500
T 481	$TPT1^+$	ND[a]	6,250
SC 761	$TPT1^+$ (P_{GAL10}-$TPT1^+$)	170,000	8,600
SC 760	$TPT1^+$	6,800	6,100
SC 839	*tpt1Δ1::LEU2* (*CEN IV* $TPT1^+$)	7,200	7,300
SC 893	*tpt1Δ1::LEU2* (P_{GAL10}-*tpt1-1*)	2,500	<110[b]

[a]Not determined.
[b]Value is below quantitation levels, which occurs within 2.5 generations after shift to glucose-containing medium; 380 units/mg were measured 1 generation after shift to glucose.

Downloaded from www.rnajournal.org on February 14, 2008

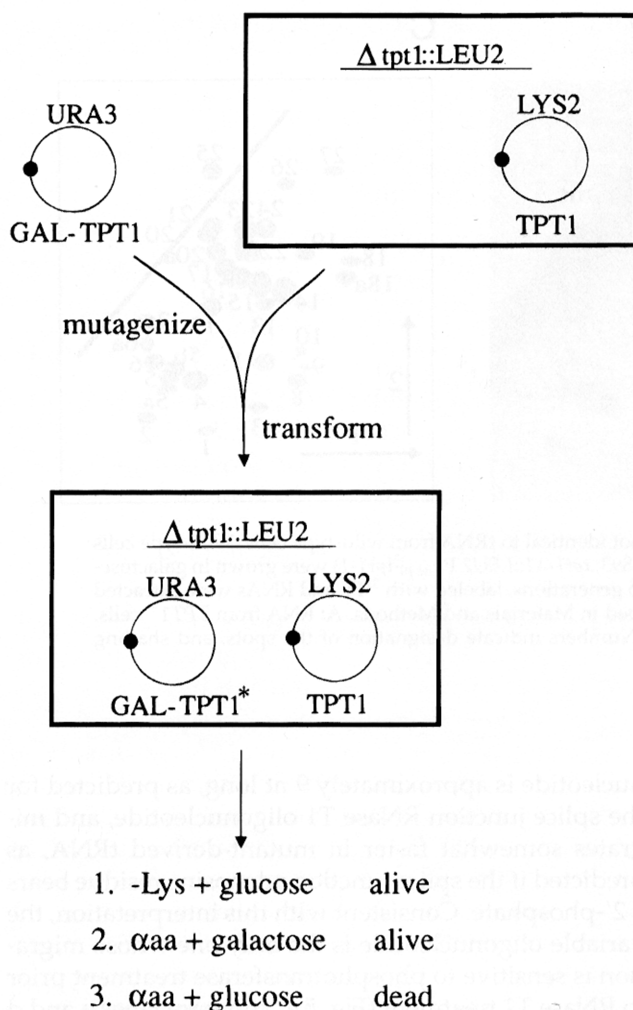

FIGURE 2. Scheme for isolation of conditional lethal *tpt1* mutants. A *URA3* plasmid containing *TPT1* under control of the *GAL10* promoter was mutagenized, transformed into a strain with its only copy of *TPT1* on a *LYS2* plasmid, and transformants were replica plated onto media containing α-aminoadipic acid and either galactose or glucose, to obtain P_{GAL10}-*tpt1* mutants, which grow on galactose but not on glucose, as described in Materials and Methods.

compared to 42,000 u/mg in extracts of SC 775, a related strain bearing the unmutagenized P_{GAL10}-*TPT1* plasmid (relevant genotype: *tpt-1Δ1::LEU2* P_{GAL10}-*TPT1*$^{+}$), and 6,000–8,000 u/mg routinely obtained from extracts of a wild-type cell (see Table 1). After shift to glucose-containing medium, the *tpt1-1* mutant is impaired for growth within 3–4 generations, when phosphotransferase activity is virtually absent (Table 1), and completely stops growing after about 8 generations. This is due to lack of Tpt1 protein, because transformation of this strain with another copy of the *TPT1* gene, with its own promoter, prevents death in glucose (data not shown). Because our goal was to investigate the consequences of a lack of 2′-phosphotransferase, the *tpt1-1* mutant provides an ideal way to study these effects.

Cells depleted for 2′-phosphotransferase accumulate tRNAs with a 2′-phosphate

To examine tRNA processing in cells depleted of Tpt1 protein, we grew P_{GAL10}-*tpt1-1* cells in galactose, shifted to glucose, and labeled with ^{32}Pi 3.5 generations later, as growth was slowing. Labeled tRNA was then displayed on two-dimensional gels and compared to tRNA from wild-type cells to look for defects in tRNA processing and splicing.

Most aspects of tRNA splicing appear to be unaffected in *tpt1-1* mutant cells grown in nonpermissive conditions. There is no obvious accumulation of either unspliced pre-tRNAs, or of tRNA half-molecules, as would be expected if the lack of Tpt1 protein impaired endonuclease or ligase function in vivo (data not shown). Similarly, as shown in Figure 3, the pattern of mature-sized tRNA produced in *tpt1-1* mutant cells is highly similar to that produced in wild-type cells, both in relative intensity and relative position of the different spots. However, there are a few distinct differences. A novel spot (18a) appears reproducibly in tRNA from *tpt1-1* mutant strains grown in nonpermissive conditions; this spot is much less intense in *TPT1*$^{+}$ cells (compare Fig. 3A and B) and in *tpt1-1* mutants grown in permissive conditions (data not shown). The same is true of spot 6a. Conversely, spot 8 is much less intense in tRNA from *tpt1-1* mutants grown in nonpermissive conditions than in tRNA from *TPT1*$^{+}$ cells (Fig. 3) or from *tpt1-1* mutants grown in permissive conditions (data not shown). These differences suggest that *tpt1-1* cells may, as predicted, have altered tRNAs bearing a 2′-phosphate.

tRNA from *tpt1-1* cells growing in nonpermissive conditions is a substrate for 2′-phosphotransferase. To demonstrate this, we eluted tRNA in batch from two-dimensional gels and assayed with phosphotransferase protein to determine if the tRNA could be used to produce Appr>p. As shown in Figure 4, phosphotransferase treatment of tRNA from *tpt1-1* mutant cells produces material comigrating with Appr>p (Fig. 4, compare lane j with lanes b and c), whereas phosphotransferase treatment of wild-type tRNA does not (Fig. 4, compare lanes f and j). As expected, formation of material comigrating with Appr>p requires both NAD and TPTase (Fig. 4, compare lanes h and i to lane j), and the product Appr>p is phosphatase resistant (data not shown). Because 2′-phosphotransferase is specific for internal 2′-phosphates (McCraith & Phizicky, 1990; Zillman et al., 1992), we conclude that *tpt1-1* mutant-derived tRNAs are 2′-phosphorylated.

A crude calculation suggests that most of the spliced tRNAs that are made at this point are 2′-phosphorylated. The amount of Appr>p that is made from batch-isolated tRNA corresponds to about 0.1% of the labeled tRNA. Because roughly 1/10 of the tRNAs in the cell contain introns, this is the expected result if

Downloaded from www.rnajournal.org on February 14, 2006

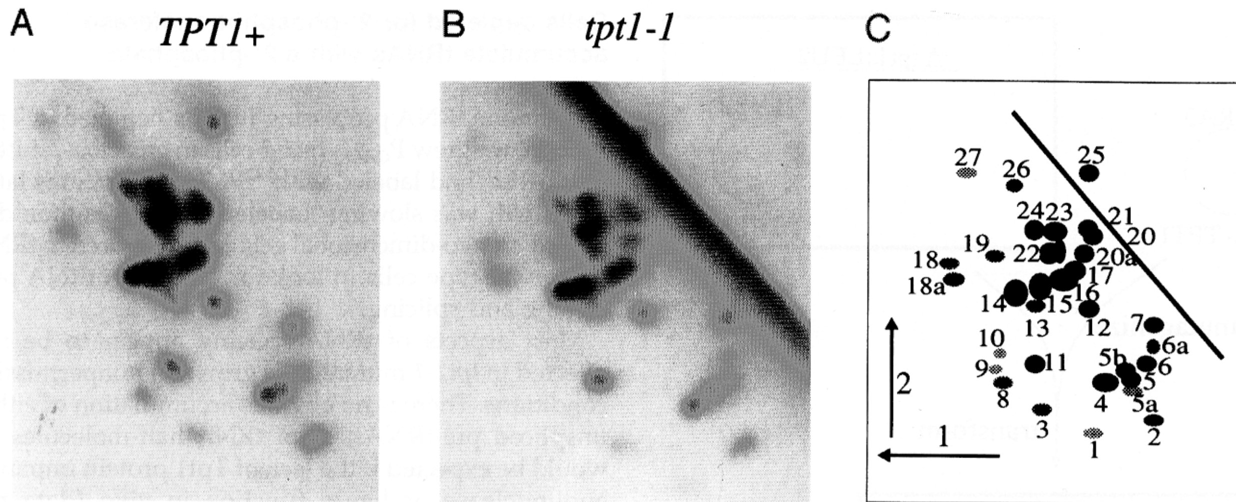

FIGURE 3. tRNA from cells lacking Tpt1 protein is similar but not identical to tRNA from wild-type cells. Wild-type cells (SC 839; *tpt1-Δ1::LEU2* P_{GAL10}-*TPT1*$^+$) and *tpt1-1* mutant cells (SC 893; *tpt1-Δ1::LEU2* P_{GAL10}-*tpt1-1*) were grown in galactose-containing medium, shifted to glucose-containing medium for 3.5 generations, labeled with ^{32}Pi, and RNAs were extracted and resolved on two-dimensional polyacrylamide gels as described in Materials and Methods. **A:** RNA from *TPT1*$^+$ cells. **B:** RNA from *tpt1-1* cells. **C:** Schematic of the resolved RNAs. Numbers indicate designation of the spots, and shading indicates relative intensity.

most or all of the spliced tRNA retained a 2′-phosphate. This is further substantiated below.

Nine individual tRNA species were able to serve as substrates for the phosphotransferase protein. To demonstrate this, each individual spot from the two-dimensional gels was eluted and assayed for Appr>p production in the presence of phosphotransferase by the same method as shown in Figure 4. Each substrate tRNA was then identified by hybridization to a set of immobilized tRNA gene sequences encompassing 9 of the 10 intron-containing tRNA gene families ($tRNA^{SER}_{UGA}$ was not used). In this way, we were able to identify eight of the nine tRNAs (see Table 2). These assignments are further supported by the size of the RNAs, RNase T1 digestion patterns, and modified nucleotide analysis (see below and data not shown). The ninth substrate, spot 23, is presently unidentified, although it is likely composed of one or more tRNA species. As expected of tRNA, modified nucleotide analysis of spot 23 shows the presence of ribothymidine, dihydrouridine, and pseudouridine, and quantitation indicates that ribo T is at its expected level of 1.3%.

2′-Phosphate is on the predicted oligonucleotide

RNase T1 digestion of $tRNA^{TYR}$ confirms that the expected oligonucleotide is phosphorylated. As shown in Figure 5A, the RNase T1 pattern for $tRNA^{TYR}$ (spot 12) from *TPT1*$^+$ and *tpt1-1* mutant cells is identical except for one oligonucleotide (Fig. 5A, compare band A in lane a to band C in lane e). This variable oligonucleotide is approximately 9 nt long, as predicted for the splice junction RNase T1 oligonucleotide, and migrates somewhat faster in mutant-derived tRNA, as predicted if the splice junction adenosine residue bears a 2′-phosphate. Consistent with this interpretation, the variable oligonucleotide is the only one whose migration is sensitive to phosphotransferase treatment prior to RNase T1 treatment (Fig. 5A, compare lanes a and d to e and h). As expected, the mobility of the mutant-derived splice junction oligonucleotide is slowed by removal of the 2′-phosphate (Fig. 5A, compare lanes e and h); however, the oligonucleotide still migrates slightly faster than expected, to position B instead of position A (Fig. 5A, compare lanes d and h). This discrepancy is addressed below.

Rnase T1 analysis of $tRNA^{LEU}_{CAA}$ yields similar conclusions. As shown in Figure 5B, the RNase T1 pattern of $tRNA^{LEU}_{CAA}$ from *tpt1-1* mutants is identical to that from wild type, except that a larger RNase T1 oligonucleotide (labeled 12-mer) has replaced the predicted 7-mer and 5-mer species that border the splice junction guanosine residue (Fig. 5B, compare lanes a and c). This is the expected result if $tRNA^{LEU}_{CAA}$ from mutant cells retains its splice junction 2′-phosphate, because RNase T1 cannot cleave at G residues with a modification at the 2′-OH position. As predicted, treatment of mutant-derived $tRNA^{LEU}_{CAA}$ with TPTase prior to RNase T1 treatment results in the disappearance of the 12-mer and the appearance of the expected 5-mer and 7-mer (Fig. 5B, see lane d); however, the new 7-mer band appears to be composed of two slightly different-sized bands, designated 7A and 7B. This discrepancy is explained below.

Downloaded from www.rnajournal.org on February 14, 2006

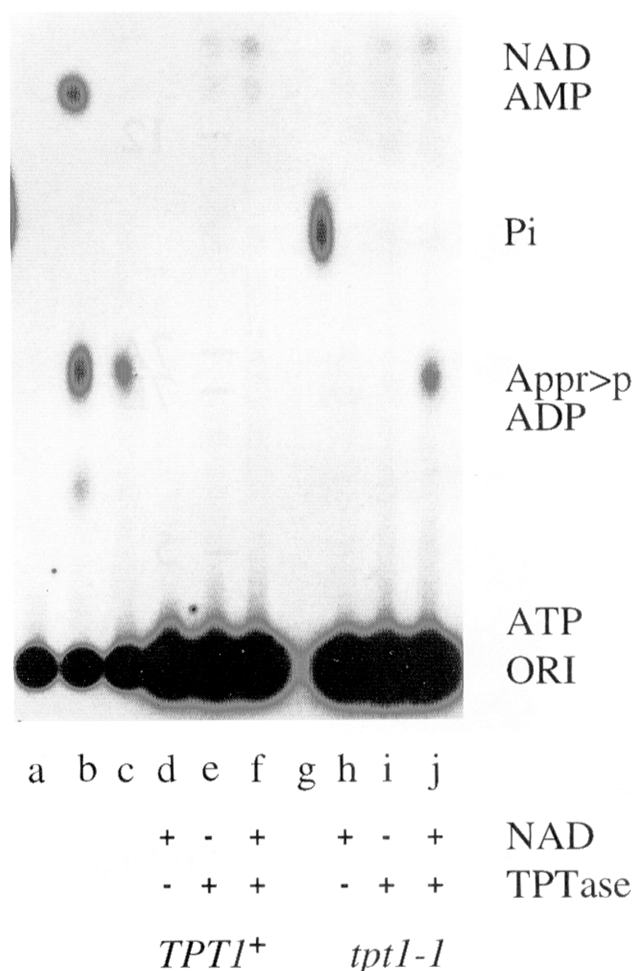

FIGURE 4. tRNA from cells lacking Tpt1 protein is a substrate for 2′-phosphotransferase. [32]Pi-labeled tRNAs from *TPT1*^{+} cells and from *tpt1-1* mutant cells growing in nonpermissive conditions were resolved on two-dimensional gels as shown in Figure 3, and batch-eluted tRNA was assayed as a substrate for 2′-phosphotransferase, as described in Materials and Methods. Reaction mixtures were applied to a polyethyleneimine cellulose thin-layer plate and developed in buffer containing 2 M sodium formate, pH 3.5. a–c: In vitro-transcribed and spliced tRNA, treated with NAD and (a) no protein; (b) partially purified yeast TPTase; (c) partially purified TPTase produced in *E. coli*. d–f, h–k: In vivo-labeled tRNA from TPT1^{+} cells (d–f) and from *tpt1-1* cells (h–k) treated with TPTase and NAD, as indicated; (g) Pi standard. Migration of standards is indicated at the right.

$tRNA^{TYR}$ and $tRNA^{LEU}_{CAA}$ from *tpt1-1* mutants are depleted for modification at the splice junction residue

The most likely explanation for the discrepancy in the mobilities of the splice junction RNase T1 oligonucleotides after removal of the 2′-phosphate is that there is a modification difference between tRNAs from wild-type and *tpt1-1* mutant cells. To investigate this directly, we purified the splice junction RNase T1 oligonucleotide of $tRNA^{TYR}$ after phosphotransferase treatment, and analyzed the modified nucleotides by RNase T2 digestion and thin layer chromatography. As shown in Figure 6, the oligonucleotide from mutant-derived $tRNA^{TYR}$ is missing an i^6A modification that is readily detected in the corresponding wild-type oligonucleotide. Quantitation of the data from this oligonucleotide demonstrates that only 0.03 mol of i^6A are present in the mutant-derived splice junction oligonucleotide, compared to 0.55 mol in wild type (data not shown). Thus, the presence of i^6A in wild-type $tRNA^{TYR}$, but not in mutant-derived $tRNA^{TYR}$, accounts for the altered mobility of the splice junction RNase T1 oligonucleotide that persists after removal of the 2′-phosphate (Fig. 5A, lanes d and h). To determine if there are other differences between the wild-type and mutant-derived $tRNA^{TYR}$ molecules, we also examined modified nucleotides of the entire $tRNA^{TYR}$ molecule by two-dimensional thin layer chromatography. As shown in Table 3, i^6A is the only nucleotide that is present at substantially lower than expected levels in mutant-derived $tRNA^{TYR}$ (0.03 mol, compared to 0.64 mol in wild-type-derived tRNA). Almost all of the other modifications are present at similar amounts in $tRNA^{TYR}$ isolated from both sources, and are present at close to the expected levels after accounting for the A^ppA splice junction dinucleotide in mutant-derived tRNA (except for m^1A plus $m^2{}_2G$, which are partially obscured, and detected at lower levels in both tRNAs). Because i^6A is normally found in $tRNA^{TYR}$ at the residue that bears the 2′-phosphate, our finding suggests that presence of the 2′-phosphate impairs modification only in the region immediately adjacent to it.

TABLE 2. Individual TPTase substrates identified by hybridization.

Spot #	Identification
5	$tRNA^{LEU}_{UAG}$
6	$tRNA^{SER}$
7	$tRNA^{LEU}_{CAA}$
12	$tRNA^{TYR}$
13	$tRNA^{LYS}$
18a	$tRNA^{PRO1}$
19	$tRNA^{PRO2}$
20a	$tRNA^{PHE}$
23	?

Because the splice junction phosphate in different tRNAs is always at the hypermodified nucleotide position (one base 3′ of the anticodon), it is almost always a modified base in mature tRNA, albeit with different modifications in different tRNAs. To determine if undermodification of the splice junction nucleotide was more general, we examined the modification state of other 2′-phosphorylated tRNAs. As shown in Figure 7, *tpt1-1*-derived $tRNA^{LEU}_{CAA}$ is clearly missing an m^1G residue, which is found in wild type only at the splice junction residue. Quantitation indicates that there are 0.2 mol of m^1G in mutant-derived $tRNA^{LEU}_{CAA}$, compared to 1.2 mol found in *TPT1*^{+}-

Downloaded from www.rnajournal.org on February 14, 2006

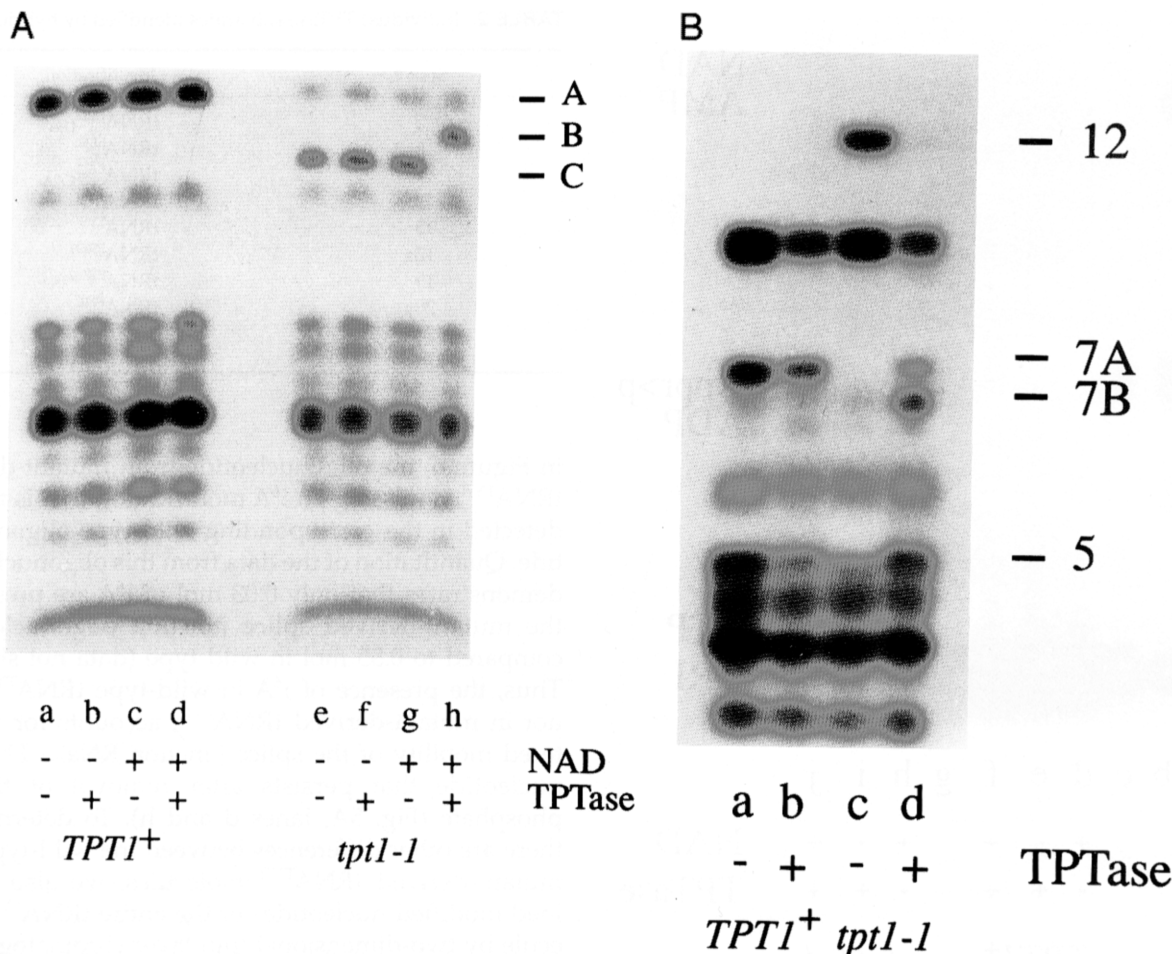

FIGURE 5. RNase T1 analysis of individual tRNAs from wild-type and *tpt1-1* mutant cells. ^{32}P-labeled tRNA from isolated spots was treated with TPTase or buffer, followed by digestion with RNase T1, and electrophoresis on a 20% polyacrylamide–7 M urea gel, as described in Materials and Methods. **A:** RNase T1 analysis of tRNA^{TYR}. tRNA^{TYR} (spot 12) isolated from either *TPT1*$^+$ (a–d) or from *tpt1-1* cells (e–h), was treated with TPTase and/or NAD as indicated, and analyzed after RNase T1 treatment. A, B, C, different mobilities of the splice junction 9-mer oligonucleotide. **B:** RNase T1 analysis of tRNA$^{LEU}{}_{CAA}$. tRNA$^{LEU}{}_{CAA}$ (spot 7) isolated from either *TPT1*$^+$ (a and b) or *tpt1-1* cells (c and d), was treated with buffer (a and c), or with NAD and TPTase (b and d), and analyzed after RNase T1 treatment. 12, 7A, 7B, and 5 represent the expected size of the splice junction RNase T1 oligonucleotides, with or without the splice junction G containing a 2′-phosphate.

derived tRNA (Table 4). This data is consistent with the distribution of 7-mer RNase T1 bands observed in mutant-derived tRNA$^{LEU}{}_{CAA}$ after dephosphorylation (Fig. 5B, bands 7A and 7B), assuming that the upper band corresponds to the m^{1}G-modified oligonucleotide and that the lower band corresponds to the oligonucleotide lacking m^{1}G. Similar results were obtained with tRNA$^{LEU}{}_{UAG}$ and tRNA^{PRO1}, both of which also have m^{1}G at the splice junction residue. For tRNA$^{LEU}{}_{UAG}$, 0.2 mol m^{1}G is observed in mutant-derived tRNA, compared to 0.9 mol m^{1}G found in the wild-type-derived tRNA (Table 4). For tRNA^{PRO1} (Fig. 3, spot 18a), there is no visible corresponding tRNA from wild-type cells with which to compare its modified nucleotide composition; for this species, the modification state of the splice junction nucleotides was assessed by comparison of the nucleotides before and after removal of the splice junction 2′-phosphate. As shown in Table 4, the splice junction residue, m^{1}G, is undermodified, because it is present at similar levels both before and after dephosphorylation (1.1 and 1.0 mol). By contrast, the residue just 3′ of the splice junction, ψ, is likely modified in the mutant-derived tRNA, because its levels increase from 2.0 to 3.2 after dephosphorylation (although the expected value is 4). We conclude that tRNA^{PRO1}, like the other tRNAs examined, is substantially less modified at the splice junction residue in *tpt1-1*-derived tRNAs than expected for wild-type-derived tRNAs. Moreover, for each tRNA that was examined, almost all of the resolved modifications were present near the values expected for mature tRNAs, including 2′-O-methylated nucleotides (Table 4, data not shown), which is discussed below. The only obvious exceptions were the absence of one of the four expected Ψ residues from spot 18a (tRNA^{PRO1}), and the modest underrepresentation of

Downloaded from www.rnajournal.org on February 14, 2006

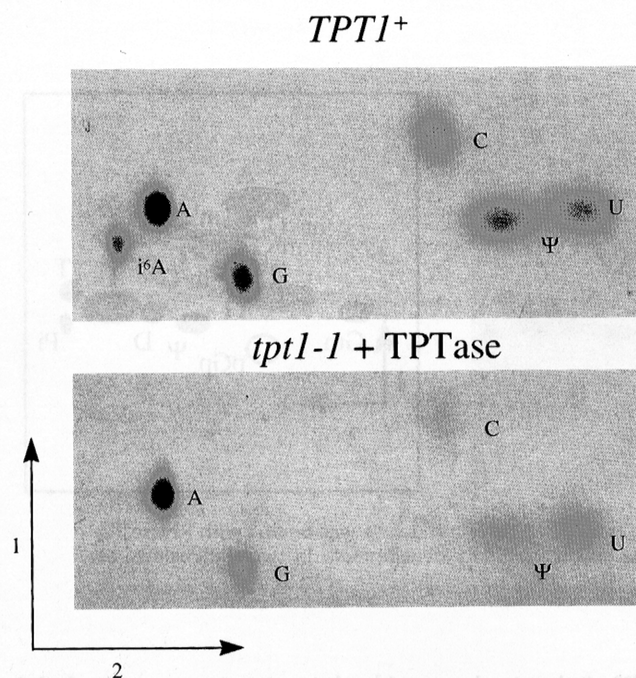

FIGURE 6. Nucleotide composition of the splice junction RNase T1 oligonucleotide of $tRNA^{TYR}$. The splice junction RNase T1 oligonucleotide of $tRNA^{TYR}$ from *tpt1-1* cells and from *TPT1*$^+$ cells was resolved on a 20% gel after treatment of the tRNA with TPTase and then RNase T1, and the eluted oligonucleotide was treated with RNase T2, applied to a PEI-cellulose thin-layer plate, and nucleotides were resolved by development in two dimensions, as described in Material and Methods.

TABLE 3. Nucleotide composition of $tRNA^{TYR}$ from *TPT1*$^+$ and *tpt1-1* mutant cells.

Nucleotide	Expected moles Pi	*TPT1*$^{+a}$	*tpt1-1*^{a}	*tpt1-1* + Tptase^a
i^6A	1	0.64	0.02	0.03
N^ppN'	3 (*tpt1-1*)	0	2.8	0
A	15	15	14	17
C	19	20	19	20
G	19	19	20	21
U	7	7.3	6.6	6.7
D	6	5.2	5.6	5.5
ψ + pC	5	4.8	4.2	4.1
T	1	1.0	1.5	0.7
m^5C	1	0.9	0.9	0.6
$m^1A + m^2{}_2G$	2	0.9^b	0.7^b	1.0^b
m^2G	1	0.9	0.7	0.6
Gm	2	1.9	1.7	1.6

[a]Moles of nucleotide calculated as (radioactivity in spot/total radioactivity) × number of phosphate residues in tRNA.
[b]Partially masked by C and A.

m^2G (about 55–70% of the expected value) consistently observed in $tRNA^{LEU}{}_{CAA}$, $tRNA^{LEU}{}_{UAG}$, and $tRNA^{TYR}$ from *tpt1-1* cells, compared to 85–95% observed in wild-type cells.

DISCUSSION

The results in this paper provide strong evidence that yeast Tpt1 protein is responsible for the last step of tRNA splicing in vivo: removal of the splice junction 2′-phosphate from ligated tRNA. Under nonpermissive conditions, a conditional *tpt1* mutant accumulates at least nine different tRNA species, each of which appears to have a splice junction 2′-phosphate. Each tRNA species is a substrate for the 2′-phosphotransferase encoded by *TPT1*, which is itself highly specific for RNA substrates with internal 2′-phosphates (McCraith & Phizicky, 1990; Zillman et al., 1992); eight of the nine substrate tRNAs were identified as tRNAs from known families of intron-containing tRNAs (Table 2); two of the substrates, $tRNA^{TYR}$ and $tRNA^{LEU}{}_{CAA}$, were shown to bear the 2′-phosphate on the expected RNase T1 oligonucleotide (Fig. 5); and, for one of these, $tRNA^{LEU}{}_{CAA}$, the 2′-phosphate is on the expected guanosine residue, because it is missing an RNase T1 site that is restored correctly after dephosphorylation (Fig. 5B). Because each of three other tRNA substrates examined, as well as $tRNA^{LEU}{}_{CAA}$, has an undermodified splice junction residue but otherwise near normal modification levels, it seems highly probable that the 2′-phosphate is located at the expected splice junction position for these tRNAs, too. We infer, based on this analysis, that the nine tRNA species identified as substrates for TPTase all bear their 2′-phosphate at the expected splice junction. The involvement of Tpt1 protein in the dephosphorylation step of tRNA splicing of most (and presumably all) intron-containing tRNAs confirms previous experiments demonstrating a single tRNA ligase involved in the joining step in vivo (Phizicky et al., 1992) and a single endonuclease acting in vitro (Peebles et al., 1983) and in vivo (Ho et al., 1990) on multiple intron-containing tRNA substrates.

The accumulation of 2′-phosphorylated tRNAs in the *tpt1-1* mutant also reaffirms part of the tRNA ligation mechanism shown in Figure 1. The mechanism of yeast tRNA ligase deduced in vitro requires opening of the cyclic phosphate to a 2′-phosphate, an exogenous source of the splice junction phosphate, and the presence of the terminal 2′-phosphate during ligation (Knapp et al., 1979; Greer et al., 1983; Westaway et al., 1993). The accumulation of 2′-phosphorylated tRNAs in *tpt1* mutants is consistent with this mechanism, and clearly different from the mechanism of the HeLa-type ligase, in which the cyclic phosphate at the end of the 5′-half-molecule is used for the splice junction phosphodiester bond.

The absence of the modification of the splice junction nucleotide in *tpt1-1* cells is strikingly general, and suggests that modification at this position normally occurs after the last step of tRNA splicing. In each of

Downloaded from www.rnajournal.org on February 14, 2006

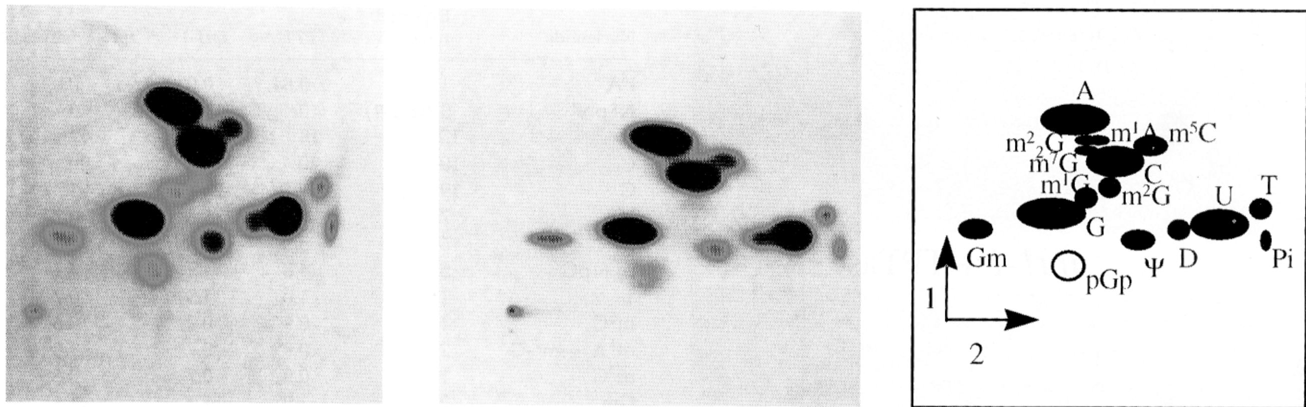

FIGURE 7. Nucleotide composition of $tRNA^{LEU}_{CAA}$. $tRNA^{LEU}_{CAA}$ from *TPT1*$^+$ and *tpt1-1* cells was treated with RNase T2, and nucleotides were applied to a PEI-cellulose thin-layer plate and resolved by development in two dimensions, as described in Materials and Methods.

the four tRNAs examined, this modification was significantly depleted, whether the nucleotide was i^6A or m^1G (Table 4). A preliminary examination of two other 2′-phosphorylated tRNAs from *tpt1-1* cells, although not accompanied by wild-type controls, extends this observation to other modifications at this position. For $tRNA^{LYS}$, the normal splice junction modification, t^6A, is not observed either before or after removal of the splice junction phosphate; similarly, $tRNA^{PHE}$ is missing the Wye base after dephosphorylation (data not shown). This result is consistent with the lack of modification at this position, which is observed in unspliced pre-tRNAs from *rna1-1* cells (Knapp et al., 1978; Etcheverry et al., 1979), with the appearance of i^6A only in mature-sized $tRNA^{TYR}$ after microinjection of *Xenopus* oocytes with the tRNA gene (Nishikura & De Robertis, 1981), and with the preferential formation of m^1G37 in mature tRNA rather than in unspliced pre-tRNA that is observed both in vitro in yeast (Strobel & Abelson, 1986) and in vivo in *Xenopus* oocytes (Grosjean et al., 1990). This absence of modification of the splice junction residue implies directly that removal of the splice junction phosphate precedes modification at the hypermodified position, regardless of the modification. By contrast, two other modifications (2′-O-methylation and m^2G) are not observed in pre-tRNAs that accumulate in *rna1-1* cells, and are nearly normal or only slightly reduced in 2′-phosphorylated tRNAs from *tpt1-1* cells. One explanation of this observation is that 2′-O-methylation and m^2G formation cannot occur before excision of the intron, but can occur on mature-sized tRNA, with or without a 2′-phosphate.

The simplest explanation of the specific undermodification of the splice junction residue observed in tRNAs from *tpt1-1* mutants is that the presence of the 2′-phosphate impedes the action of the modification en-

TABLE 4. Selected nucleotide composition of 2′-phosphorylated tRNAs from *tpt1-1* mutants.

tRNA	Splice junction	Nucleotide	Expected moles Pi	*TPT1*$^+$[a]	*tpt1-1*[a]	*tpt1-1* + TPTase[a]
$tRNA^{TYR}$	i^6A^ppAp	i^6A	1	0.6	0.02	0.03
		Gm	2	1.9	1.7	1.6
$tRNA^{LEU}_{CAA}$	$m^1G^pпCp$	m^1G	1	1.2	ND[b]	0.2
		m^2G	1	0.8	ND	0.5
		Gm	2	2.0	ND	2.1
$tRNA^{LEU}_{UAG}$	m^1G^ppCp	m^1G	1	0.9	0.07	0.20
		m^2G	1	1.2	0.7	0.5
		Gm	2	1.8	3.0	2.4
$tRNA^{PRO1}$	$m^1G^pp\psi p$	m^1G	2	ND	1.1	1.0
		ψ	4	ND	2.0	3.2
		Cm	2	ND	1.9	2.0

[a]Moles of nucleotide calculated as in Table 3.
[b]Not determined.

Downloaded from www.rnajournal.org on February 14, 2006

zymes that act there. The presence of a 2′-phosphate between the last two adenosine residues could prevent formation of i^6A, because the *Escherichia coli* enzyme that catalyzes i^6A formation (Δ2-isopentenyl pyrophosphate:tRNA isopentenyl transferase; IPP transferase) is highly sensitive to the sequence and structure of the anticodon stem-loop, recognizing A36-A37-A38 and other structural features in the adjacent stem (Carbon & Fleck, 1974; Yarus et al., 1986; Motorin et al., 1997). Similarly, m^1G formation at the hypermodified position of $tRNA^{PRO}{}_{GGG}$ is highly specific for structure in vitro in *E. coli* (Holmes et al., 1992) and in vivo in both *Salmonella typhimurium* (Qian & Bjork, 1997) and *Xenopus* oocytes (Grosjean et al., 1996). The effect of the 2′-phosphate on tRNA structure and recognition by the modification enzymes has not yet been explored. An alternative explanation of the missing modifications at the splice junction residue is that phosphotransferase, like ligase (Clark & Abelson, 1987), is located in the nucleus, and that the presence of the 2′-phosphate prevents the tRNA from being transported to the cytoplasm for modification at the hypermodified position. This is improbable in the case of i^6A modification, because the yeast IPP transferase that catalyzes this reaction is found in both the cytoplasm and the nucleus (Boguta et al., 1994).

Spot 18a, which corresponds to $tRNA^{PRO1}$, may be an altered conformation of the normal $tRNA^{PRO1}$. Spot 18a is unique in that its relative abundance is distinctly greater in *tpt1-1* mutants than in wild-type cells (Fig. 3). Furthermore, of the substrate species we have identified, this is the only one whose 2′-phosphate was detectable only after denaturing the tRNA. Whereas each of the other substrate tRNAs is dephosphorylated efficiently by 1 unit of phosphotransferase, no dephosphorylation of spot 18a was observed with 1,300 units in any of the three preparations characterized. However, when spot 18a was denatured prior to assay, by boiling and quick chilling, dephosphorylation was readily detected (data not shown). One reasonable interpretation of these observations is that spot 18a represents a stable alternative conformation of $tRNA^{PRO1}$ bearing a 2′-phosphate. Spot 8 may be the normal $tRNA^{PRO1}$ species, because it disappears when Tpt1 protein is limiting, concomitant with the appearance of spot 18a (Fig. 3), and its RNase T1 pattern is virtually identical to that of spot 18a. An altered conformation of $tRNA^{PRO1}$ could arise during isolation of the tRNA, or as a consequence of the 2′-phosphorylation of this tRNA species. There is evidence that altered conformations can occur in the absence of Mg^{2+} (Hall et al., 1989), which occurs during isolation of the tRNAs, and that base modifications can influence the conformations of tRNA (Derrick & Horowitz, 1993). It is therefore reasonable to speculate that 2′-phosphorylated $tRNA^{PRO1}$ lacking its splice junction m^1G is in an altered conformation. Indeed, the tRNA substrates we have not identified may also be in an alternative conformation, requiring denaturation to detect their 2′-phosphate.

Because Tpt1 protein is essential in yeast, and because *tpt1-1* mutants accumulate a number of different 2′-phosphorylated tRNAs that are depleted for modification at the hypermodfied residue, it seems likely that the defect in tRNA processing is the lethal defect. Failure to modify nucleotides at the hypermodified position can affect translational fidelity in both *S. typhimurium* (Esberg & Bjork, 1995) and *S. cerevisiae* (Laten et al., 1978; Dihanich et al., 1987), as well as growth rate in *S. typhimurium* mutants that do not make m^1G (Bjork et al., 1989). Thus, it is likely that cells with a number of tRNAs bearing 2′-phosphates at position 37, and also lacking the modification at this site, will have a serious translation defect. We have not yet ascertained the effect of these tRNAs on steps of translation.

In addition to its role in tRNA processing, as documented here, there are two other possible functions of Tpt1 protein that are unexplored. First, lack of Tpt1 protein may affect the *HAC1*-mediated unfolded protein response. This possibility stems from the startling discovery that yeast tRNA ligase is implicated genetically in the splicing of *HAC1* mRNA (Sidrauski et al., 1996). If yeast tRNA ligase does indeed splice *HAC1* mRNA, it may acquire a 2′-phosphate at its splice junction, which would be a potential substrate for Tpt1 protein. Second, lack of Tpt1 protein also ought to lead to lack of Appr>p because, to our knowledge, 2′-phosphotransferase activity is the only known origin of Appr>p in the cell. We have speculated previously that Appr>p (or one of its downstream products) may have an important cellular role, based in part on the fact that it is produced in yeast once every time tRNA is spliced, and in part on the known regulatory roles of other small molecules containing cyclic phosphates (Culver et al., 1997).

MATERIALS AND METHODS

Strains and plasmids

Strains used in this study are summarized in Table 5. SC839 (*MATα ura3-52, leu2-3,112, ade2-101, his3-Δ200, trp1-Δ901, tpt1-Δ1::LEU2* [pEMP 1062 (*CEN IV, TRP1, LYS2, TPT1*$^+$)] is the starting strain for screening for *tpt1* mutants by plasmid shuffle methods, and was made as follows. T481 was crossed with SEY6210, and its spore T881 was backcrossed twice with T581 (a *MATα LYS2, his3-Δ200* isogenic derivative of T481), from the second of which two haploid spores were crossed to give the diploid SC710. SC739 (*TPT1*$^+$/*tpt1-Δ1::LEU2*) was derived from SC710 by one-step gene replacement with pGMC22 (*tpt1-Δ1::LEU2*). SC739 was transformed with pGMC1 (*URA3, CEN IV, TPT1*$^+$) and then dissected to yield SC751 (*tpt1-Δ1::LEU2*, [pGMC1 (*URA3, CEN IV, TPT1*$^+$)], and a *lys2*$^-$ derivative (SC814) was made by selection on plates containing α-aminoadipic acid (Sikorski &

Downloaded from www.rnajournal.org on February 14, 2006

TABLE 5. Strains used in this study.

Strain	Genotype	Source
T481	*MATa ura-3-52, ade2-101, lys2-801*	(Phizicky et al., 1992)
T581	*MATα ura-3-52, ade2-101, LYS2⁺ his3-Δ200*	This study
SEY 6210	*MATα, ura3-52, leu2-3,112, his3-Δ200, trp1-901, lys2-801, suc2-Δ9,* Mel⁻ Gal⁺	(Robinson et al., 1988)
SC466	*MATα ura-3-52, his3-Δ200, lys2-801, leu2-3,112*	(Robinson et al., 1994)
SC760	SC466 [pBM258- (*CEN IV URA3* P_{GAL10})]	This study
SC761	SC466 [pGMC21- (*CEN IV URA3* P_{GAL10}-*TPT1⁺*)]	This study
SC763	*SC466 [pEMP1019- (2μ URA3* P_{GAL}-GST-TPT1)]	This study
T881	*MATa, ura3-52, ade2-101, his3-Δ200, trp1-901, lys2-801, leu2-3,112,* Gal⁺	spore of T481 X SEY6210
D180a-6d	*MATa ura-3-52, ade2-101, his3-Δ200, trp1-901, lys2-801, leu2-3,112*	spore of T881 X T581
SC767	*MATα ura-3-52, ade2-101, leu2-3,112, his3-Δ200*	spore of D180a-6d X T581
SC770	*MATa ura-3-52, ade2-101, trp1-901, lys2-801, leu2-3,112*	spore of D180a-6d X T581
SC710	SC767 X SC770	This study
SC739	SC710 *tpt1-Δ1::LEU2/TPT1⁺*	This study
SC751	*MATα ura3-52, leu2-3,112, ade2-101, his3-Δ200 trp1-Δ901 tpt1-Δ1::LEU2,* [pGMC1 (*URA3, CEN IV, TPT1⁺*)]	spore of SC739 + pGMC1
SC814	SC751 lys2⁻	This study
SC839	SC814 *tpt1-Δ1::LEU2* [pEMP 1062 (*CEN IV TRP1 LYS2, TPT1⁺*)]	This study
SC775	*MATa ura3-52 leu2-3,112, ade2-101 lys2-801, trp1-901 tpt1-Δ1::LEU2* [pGMC21 (*CEN IV URA3* P_{GAL10}-*TPT1*)]	spore of SC739 + pGMC21
SC894	*SC839 tpt1-Δ1::LEU2* [pGMC21 (*CEN IV URA3* P_{GAL10}-*TPT1*)]	This study
SC893	SC894 [pGMC21 (*CEN IV URA3* P_{GAL10}-*tpt1-1*)]	This study

Boeke, 1991). Transformation of SC814 with pEMP 1062 (*CEN IV TRP1, LYS2, TPT1⁺*), followed by selection on 5-FOA for loss of the URA3 plasmid, yielded SC839.

SC894 (*tpt1-Δ1::LEU2* P_{GAL10}-*TPT1*) is derived from SC839, by transformation with pGMC21 and selection for loss of the *LYS2* plasmid on α-aminoadipic acid. SC893 (*tpt1-Δ1::LEU2* [P_{GAL10}-*tpt1-1*]) is a glucose-conditional *tpt1* mutant strain (abbreviated as *tpt1-1*), which is isogenic with SC 894, except that its pGMC 21 plasmid was mutagenized prior to transformation.

SC775 (*tpt1-Δ1::LEU2* [pGMC21 (*CEN IV, URA3* P_{GAL10}-*TPT1*)]) is a meiotic segregant of SC 739, after transformation with pGMC21 (*CEN IV, URA3* P_{GAL10}-*TPT1⁺*). SC466 is derived from T481 and a closely related laboratory strain.

pGMC21 (*CEN IV, URA3* P_{GAL10}-*TPT1⁺*) contains the *TPT1* gene under control of the *GAL10* promoter (Culver et al., 1997). pEMP1062 was obtained by ligation of an *Eco*R I-*Pst* I fragment of *LYS2* DNA into the vector YCP lac22 (CEN IV, TRP1) (Gietz & Sugino, 1988), followed by ligation of the *Eco*R I-*Sac* I fragment encompassing the *TPT1* gene into the *Eco*R I site of this plasmid.

Isolation of conditional *tpt1* mutants

Plasmid pGMC21 was mutagenized with hydroxylamine for 3 h, at 70° (Phizicky et al., 1992), transformed into SC839, and Ura⁺ transformants were screened for those that grew on α-aminoadipic acid plates containing galactose, but not on α-aminoadipic acid plates containing glucose. Of 500 transformants tested, 4 strains were obtained that were Gal⁺ and Glucose⁻. These strains were also α-aminoadipic acid resistant, Lys⁻, and Trp⁻, as expected for loss of the original resident plasmid, pEMP1062. All four candidates (including SC893) regained the ability to grow on glucose after transformation with a plasmid (pEMP1062) containing another copy of *TPT1*. In each case, the lesion was shown to be plasmid dependent by isolation of the plasmid in *E. coli*, and re-transformation into the starting yeast strain. Subsequently, the lesion was localized by sequencing, a fragment containing the lesion was ligated into an unmutagenized parent plasmid, and the glucose conditional phenotype was confirmed after transformation of the plasmid into SC 839.

Labeling with ^{32}Pi

Yeast strains were labeled with ^{32}Pi as described (Phizicky et al., 1992). Strain SC893 (P_{GAL10}-*tpt1-1*) and its control strain SC839 (P_{GAL10}-*TPT1⁺*) were grown overnight in YP medium containing 2% galactose, and shifted to YP medium containing 2% glucose for two generations. Cells were then harvested, washed in low-phosphate medium containing 2% glucose, resuspended at 2×10^7 cells/mL (20 mL) of this medium, grown for 1.5 more generations, and labeled for 30 min with 0.25 mCi of ^{32}Pi/mL.

Extraction, purification, and analysis of RNA

In vivo-labeled RNA was extracted from cells as described (Knapp et al., 1978), and analyzed on two-dimensional polyacrylamide gels (10%, first dimension; 20% second dimension) containing 4 M urea and TBE (88 mM Tris-borate, 1 mM EDTA, pH 8.3), as described (Phizicky et al., 1992), except that the second dimension was run for longer to resolve tRNAs. RNA and RNase T1 oligonucleotides were eluted from gels in buffer containing 0.5 M ammonium acetate, 1 mM EDTA, and 0.1% SDS, followed by phenol extraction and ethanol precipitation.

RNase T1 analysis was performed with 5 units RNase T1 and 2,000 cpm of labeled tRNA, with or without 10 μg carrier RNA, followed by electrophoresis on a 20% polyacryl-

Downloaded from www.rnajournal.org on February 14, 2006

amide-7 M urea-TBE gel, as described (Phizicky et al., 1992). RNase T2 digestion was performed as described (Gegenheimer et al., 1983), with 200–2,000 cpm of labeled RNA and 10 μg carrier RNA. RNase T2 digestions were analyzed on cellulose thin-layer plates developed in two dimensions, as described previously (Saneyoshi et al., 1972), or on polyethylenimine-cellulose plates developed in two dimensions as described (Bochner & Ames, 1982), with first-dimension solvent Aa (1 M acetic acid, pH 3.5, adjusted with ammonium hydroxide) and second-dimension solvent Sa (74 g ammonium sulfate, 0.4 g ammonium bisulfate, 100 mL H_2O). Resolved spots were quantitated on a Molecular Dynamics Phosphorimager, except for those of $tRNA^{LEU}{}_{UAG}$, which were quantitated by densitometry.

RNAs were treated with 2′-phosphotransferase (or buffer) using 1–1,300 units of enzyme. Samples that were subsequently treated with RNase T1 or RNase T2 were first extracted with phenol, precipitated with ethanol, and resuspended in TE buffer.

Hybridization of labeled tRNAs to tRNA genes

Plasmids containing tRNA genes were immobilized on nitrocellulose, after restriction digestion, by incubation in 500 μL denaturation buffer (0.2 M NaOH and 6× SSC) at 68 °C for 10 min, application to the filter, washing with 1 mL 6× SSC, and baking (Sambrook et al., 1989). Filters were pre-hybridized in buffer containing 5× SSPE, 2× Denhardt's solution, 0.1% SDS, and 50% formamide at 42 °C for 2 h, hybridized overnight at 40 °C in the same buffer containing 10,000–20,000 cpm of labeled denatured tRNAs isolated from 2D gels, washed with 1× SSC, 0.1% SDS at room temperature for 20 min, and three times with 0.2× SSC, 0.1% SDS for 20 min at 33 °C.

Assay of 2′-phosphotransferase activity

2′-Phosphotransferase activity was measured as described (Culver et al., 1997), with partially purified 2′-phosphotransferase, 1 mM NAD, and either of two sources of tRNA with a labeled splice junction 2′-phosphate: $tRNA^{PHE}$ prepared by in vitro transcription with [α-^{32}P] ATP, followed by treatment with endonuclease and ligase, or uniformly labeled tRNA isolated from yeast cells labeled with ^{32}Pi, after elution from two-dimensional polyacrylamide gels.

2′-phosphotransferase protein

TPTase was obtained from yeast or *E. coli* strains containing expression plasmids. *E. coli* strain RZ510 contains pGMC9, in which expression of the *TPT1* gene is under control of the *tac* promoter (Culver et al., 1997); its TPTase was partially purified from extracts of IPTG-induced cells, by Blue Sepharose column chromatography, essentially as described (Culver et al., 1997). A GST-Tpt1 fusion protein was purified by glutathione agarose chromatography from yeast strain SC763, after induction of synthesis of the fusion protein with medium containing galactose.

ACKNOWLEDGMENTS

We thank Gloria Culver, Mark Dumont, Elizabeth Grayhack, and Matthew Robinson for valuable advice and for critical reading of the manuscript. This research was supported by National Institutes of Health grant GM52347 to E.M.P.

Received August 25, 1997; returned for revision September 7, 1997; revised manuscript received September 16, 1997

REFERENCES

Aebi M, Kirchner G, Chen JY, Vijayraghavan U, Jacobson A, Martin NC, Abelson J. 1990. Isolation of a temperature-sensitive mutant with an altered tRNA nucleotidyltransferase and cloning of the gene encoding tRNA nucleotidyltransferase in the yeast *Saccharomyces cerevisiae*. *J Biol Chem 265*:16216–16220.

Attardi DG, Margarit I, Tocchini-Valentini GP. 1985. Structural alterations in mutant precursors of the yeast tRNALeu3 gene which behave as defective substrates for a highly purified splicing endoribonuclease. *EMBO J 4*:3289–3297.

Baldi MI, Mattoccia E, Bufardeci E, Fabbri S, Tocchini-Valentini GP. 1992. Participation of the intron in the reaction catalyzed by the *Xenopus* tRNA splicing endonuclease [see comments]. *Science 255*:1404–1408.

Baldi MI, Mattoccia E, Ciafre S, Attardi DG, Tocchini-Valentini GP. 1986. Binding and cleavage of pre-tRNA by the *Xenopus* splicing endonuclease: Two separable steps of the intron excision reaction. *Cell 47*:965–971.

Belford HG, Westaway SK, Abelson J, Greer CL. 1993. Multiple nucleotide cofactor use by yeast ligase in tRNA splicing. Evidence for independent ATP- and GTP-binding sites. *J Biol Chem 268*:2444–2450.

Bjork GR, Wikstrom PM, Bystrom AS. 1989. Prevention of translational frameshifting by the modified nucleoside 1-methylguanosine. *Science 244*:986–989.

Bochner BR, Ames BN. 1982. Complete analysis of cellular nucleotides by two-dimensional thin layer chromatography. *J Biol Chem 257*:9759–9769.

Boguta M, Hunter LA, Shen WC, Gillman EC, Martin NC, Hopper AK. 1994. Subcellular locations of MOD5 proteins: Mapping of sequences sufficient for targeting to mitochondria and demonstration that mitochondrial and nuclear isoforms commingle in the cytosol. *Mol Cell Biol 14*:2298–2306.

Carbon J, Fleck EW. 1974. Genetic alteration of structure and function in glycine transfer RNA of *Escherichia coli*: Mechanism of suppression of the tryptophan synthetase A78 mutation. *J Mol Biol 85*:371–391.

Clark MW, Abelson J. 1987. The subnuclear localization of tRNA ligase in yeast. *J Cell Biol 105*:1515–1526.

Culver GM, McCraith SM, Consaul SA, Stanford DR, Phizicky EM. 1997. A 2′-phosphotransferase implicated in tRNA splicing is essential in *Saccharomyces cerevisiae*. *J Biol Chem 272*:13203–13210.

Culver GM, McCraith SM, Zillmann M, Kierzek R, Michaud N, LaReau RD, Turner DH, Phizicky EM. 1993. An NAD derivative produced during transfer RNA splicing: ADP-ribose 1″-2″ cyclic phosphate. *Science 261*:206–208.

Derrick WB, Horowitz J. 1993. Probing structural differences between native and in vitro transcribed *Escherichia coli* valine transfer RNA: Evidence for stable base modification-dependent conformers. *Nucleic Acids Res 21*:4948–4953.

Dihanich ME, Najarian D, Clark R, Gillman EC, Martin NC, Hopper AK. 1987. Isolation and characterization of MOD5, a gene required for isopentenylation of cytoplasmic and mitochondrial tRNAs of *Saccharomyces cerevisiae*. *Mol Cell Biol 7*:177–184 [published erratum appears in *Mol Cell Biol 7*(5):2035; 1987 May].

Esberg B, Bjork GR. 1995. The methylthio group (ms2) of N6-(4-hydroxyisopentenyl)-2-methylthioadenosine (ms2io6A) present next to the anticodon contributes to the decoding efficiency of the tRNA. *J Bacteriol 177*:1967–1975.

Etcheverry T, Colby D, Guthrie C. 1979. A precursor to a minor species of yeast tRNASer contains an intervening sequence. *Cell 18*:11–26.

Filipowicz W, Konarska M, Gross HJ, Shatkin AJ. 1983. RNA 3′-

Downloaded from www.rnajournal.org on February 14, 2006

terminal phosphate cyclase activity and RNA ligation in HeLa cell extract. *Nucleic Acids Res 11*:1405–1418.

Gegenheimer P, Gabius HJ, Peebles CL, Abelson J. 1983. An RNA ligase from wheat germ which participates in transfer RNA splicing in vitro. *J Biol Chem 258*:8365–8373.

Gietz RD, Sugino A. 1988. New yeast–*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene 74*:527–534.

Greer CL, Peebles CL, Gegenheimer P, Abelson J. 1983. Mechanism of action of a yeast RNA ligase in tRNA splicing. *Cell 32*:537–546.

Grosjean H, Droogmans L, Giege R, Uhlenbeck OC. 1990. Guanosine modifications in runoff transcripts of synthetic transfer RNA-Phe genes microinjected into *Xenopus* oocytes. *Biochim Biophys Acta 1050*:267–273.

Grosjean H, Edqvist J, Straby KB, Giege R. 1996. Enzymatic formation of modified nucleosides in tRNA: Dependence on tRNA architecture. *J Mol Biol 255*:67–85.

Hall KB, Sampson JR, Uhlenbeck OC, Redfield AG. 1989. Structure of an unmodified tRNA molecule. *Biochemistry 28*:5794–5801.

Ho CK, Rauhut R, Vijayraghavan U, Abelson J. 1990. Accumulation of pre-tRNA splicing "2/3" intermediates in a *Saccharomyces cerevisiae* mutant. *EMBO J 9*:1245–1252.

Holmes WM, Andraos-Selim C, Roberts I, Wahab SZ. 1992. Structural requirements for tRNA methylation. Action of *Escherichia coli* tRNA(guanosine-1)methyltransferase on tRNA(1Leu) structural variants. *J Biol Chem 267*:13440–13445.

Hopper AK, Banks F. 1978. A yeast mutant which accumulates precursor tRNAs. *Cell 14*:211–219.

Hopper AK, Martin NC. 1992. Processing of yeast cytoplasmic and mitochondrial precursor tRNAs. In: Jones EW, Pringle JR, Broach JR, eds. *The molecular and cellular biology of the yeast Saccharomyces: Gene expression*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 99–141.

Knapp G, Beckmann JS, Johnson PF, Fuhrman SA, Abelson J. 1978. Transcription and processing of intervening sequences in yeast tRNA genes. *Cell 14*:221–236.

Knapp G, Ogden RC, Peebles CL, Abelson J. 1979. Splicing of yeast tRNA precursors: Structure of the reaction intermediates. *Cell 18*:37–45.

Konarska M, Filipowicz W, Domdey H, Gross HJ. 1981. Formation of a 2′-phosphomonoester, 3′,5′-phosphodiester linkage by a novel RNA ligase in wheat germ. *Nature 293*:112–116.

Laski FA, Fire AZ, RajBhandary UL, Sharp PA. 1983. Characterization of tRNA precursor splicing in mammalian extracts. *J Biol Chem 258*:11974–11980.

Laten H, Gorman J, Bock RM. 1978. Isopentenyladenosine deficient tRNA from an antisuppressor mutant of *Saccharomyces cerevisiae*. *Nucleic Acids Res 5*:4329–4342.

Lee JY, Rohlman CE, Molony LA, Engelke DR. 1991. Characterization of RPR1, an essential gene encoding the RNA component of *Saccharomyces cerevisiae* nuclear RNase P. *Mol Cell Biol 11*:721–730.

Lee MC, Knapp G. 1985. Transfer RNA splicing in *Saccharomyces cerevisiae*. Secondary and tertiary structures of the substrates. *J Biol Chem 260*:3108–3115.

Mattoccia E, Baldi IM, Gandini-Attardi D, Ciafre S, Tocchini-Valentini GP. 1988. Site selection by the tRNA splicing endonuclease of *Xenopus laevis*. *Cell 55*:731–738.

McCraith SM, Phizicky EM. 1990. A highly specific phosphatase from *Saccharomyces cerevisiae* implicated in tRNA splicing. *Mol Cell Biol 10*:1049–1055.

McCraith SM, Phizicky EM. 1991. An enzyme from *Saccharomyces cerevisiae* uses NAD+ to transfer the splice junction 2′-phosphate from ligated tRNA to an acceptor molecule. *J Biol Chem 266*:11986–11992.

Melton DA, De Robertis EM, Cortese R. 1980. Order and intracellular location of the events involved in the maturation of a spliced tRNA. *Nature 284*:143–148.

Miao F, Abelson J. 1993. Yeast tRNA-splicing endonuclease cleaves precursor tRNA in a random pathway. *J Biol Chem 268*:672–677.

Motorin Y, Bec G, Tewari R, Grosjean H. 1997. Transfer RNA recognition by the *Escherichia coli* Δ^2-isopentenyl-pyrophosphate:tRNA Δ^2-isopentenyl transferase: Dependence on the anticodon arm structure. *RNA 3*:721–733.

Nishikura K, De Robertis EM. 1981. RNA processing in microinjected *Xenopus* oocytes. Sequential addition of base modifications in the spliced transfer RNA. *J Mol Biol 145*:405–420.

O'Connor JP, Peebles CL. 1991. In vivo pre-tRNA processing in *Saccharomyces cerevisiae*. *Mol Cell Biol 11*:425–439.

Peebles CL, Gegenheimer P, Abelson J. 1983. Precise excision of intervening sequences from precursor tRNAs by a membrane-associated yeast endonuclease. *Cell 32*:525–536.

Phizicky EM, Consaul SA, Nehrke KW, Abelson J. 1992. Yeast tRNA ligase mutants are nonviable and accumulate tRNA splicing intermediates. *J Biol Chem 267*:4577–4582.

Phizicky EM, Schwartz RC, Abelson J. 1986. *Saccharomyces cerevisiae* tRNA ligase. Purification of the protein and isolation of the structural gene. *J Biol Chem 261*:2978–2986.

Qian Q, Bjork GR. 1997. Structural requirements for the formation of 1-methylguanosine in vivo in tRNA(Pro)GGG of *Salmonella typhimurium*. *J Mol Biol 266*:283–296.

Reyes VM, Abelson J. 1988. Substrate recognition and splice site determination in yeast tRNA splicing. *Cell 55*:719–730.

Robinson JS, Klionsky DJ, Banta LM, Emr SD. 1988. Protein sorting in *Saccharomyces cerevisiae*: Isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol Cell Biol 8*:4936–4948.

Robinson MK, van Zyl WH, Phizicky EM, Broach JR. 1994. TPD1 of *Saccharomyces cerevisiae* encodes a protein phosphatase 2C-like activity implicated in tRNA splicing and cell separation. *Mol Cell Biol 14*:3634–3645.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual, 2nd ed*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Saneyoshi M, Oashi Z, Harada F, Nishimura S. 1972. Isolation and characterization of 2-methyladenosine from *Escherichia coli* tRNA Glu 2, tRNA Asp 1, tRNA His 1 and tRNA Arg. *Biochim Biophys Acta 262*:1–10.

Schwartz RC, Greer CL, Gegenheimer P, Abelson J. 1983. Enzymatic mechanism of an RNA ligase from wheat germ. *J Biol Chem 258*:8374–8383.

Sidrauski C, Cox JS, Walter P. 1996. tRna ligase is required for regulated mRna splicing in the unfolded protein response [see comments]. *Cell 87*:405–413.

Sikorski RS, Boeke JD. 1991. In vitro mutagenesis and plasmid shuffling: From cloned gene to mutant yeast. *Methods Enzymol 194*:302–318.

Strobel MC, Abelson J. 1986. Effect of intron mutations on processing and function of *Saccharomyces cerevisiae* SUP53 tRNA in vitro and in vivo. *Mol Cell Biol 6*:2663–2673.

Swerdlow H, Guthrie C. 1984. Structure of intron-containing tRNA precursors. Analysis of solution conformation using chemical and enzymatic probes. *J Biol Chem 259*:5197–5207.

Szekely E, Belford HG, Greer CL. 1988. Intron sequence and structure requirements for tRNA splicing in *Saccharomyces cerevisiae*. *J Biol Chem 263*:13839–13847.

Trotta CR, Miao F, Arn EA, Stevens SW, Ho CK, Rauhut R, Abelson JN. 1997. The yeast tRNA splicing endonuclease: A tetrameric enzyme with two active site subunits homologous to the archaeal tRNA endonucleases. *Cell 89*:849–858.

van Tol H, Beier H. 1988. All human tRNATyr genes contain introns as a prerequisite for pseudouridine biosynthesis in the anticodon. *Nucleic Acids Res 16*:1951–1966.

Westaway SK, Abelson J. 1995. Splicing of tRNA precursors. In: Soll D, RajBhandary UL, eds. *tRNA: Structure, biosynthesis and function*. Washington, DC: ASM Press. pp 79–92.

Westaway SK, Belford HG, Apostol BL, Abelson J, Greer CL. 1993. Novel activity of a yeast ligase deletion polypeptide. Evidence for GTP-dependent tRNA splicing. *J Biol Chem 268*:2435–2443.

Yarus M, Cline S, Raftery L, Wier P, Bradley D. 1986. The translational efficiency of tRNA is a property of the anticodon arm. *J Biol Chem 261*:10496–10505.

Yoo CJ, Wolin SL. 1997. The yeast La protein is required for the 3′ endonucleolytic cleavage that matures tRNA precursors. *Cell 89*:393–402.

Zillman M, Gorovsky MA, Phizicky EM. 1992. HeLa cells contain a 2′-phosphate-specific phosphotransferase similar to a yeast enzyme implicated in tRNA splicing. *J Biol Chem 267*:10289–10294.

Zillmann M, Gorovsky MA, Phizicky EM. 1991. Conserved mechanism of tRNA splicing in eukaryotes. *Mol Cell Biol 11*:5410–5416.