Partial activity is seen with many substitutions of highly conserved active site residues in human Pseudouridine synthase 1

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ABSTRACT

Pseudouridine synthase 1 (Pus1p) is an enzyme that converts uridine to Pseudouridine (Ψ) in tRNA and other RNAs in eukaryotes. The active site of Pus1p is composed of stretches of amino acids that are highly conserved and it is hypothesized that mutation of select residues would impair the enzyme's ability to catalyze the formation of Ψ . However, most mutagenesis studies have been confined to substitution of the catalytic aspartate, which invariably results in an inactive enzyme in all Ψ synthases tested. To determine the requirements for particular amino acids at certain absolutely conserved positions in Pus1p, three residues (R116, Y173, R267) that correspond to amino acids known to compose the active site of TruA, a bacterial Ψ synthase that is homologous to Pus1p, were mutated in human Pus1p (hPus1p). The effects of those mutations were determined with three different *in vitro* assays of pseudouridylation and several tRNA substrates. Surprisingly, it was found that each of these components of the hPus1p active site could tolerate certain amino acid substitutions and in fact most mutants exhibited some activity. The most active mutants retained near wild-type activity at positions 27 or 28 in the substrate tRNA, but activity was greatly reduced or absent at other positions in tRNA readily modified by wild-type hPus1p.

Keywords: pseudouridine; synthase; tRNA; modification; mutagenesis

INTRODUCTION

Pseudouridine (Ψ) is a post-transcriptionally modified form of uridine found in stable RNAs such as ribosomal RNA (rRNA), transfer RNA (tRNA), and small nuclear RNA (snRNA) (Bjork et al. 1987; Massenet et al. 1998; Ofengand 2002). It is one of the most abundant modified nucleotides and the positions of Ψ residues within RNAs are well conserved (Auffinger and Westhof 1998). Pseudouridine plays an important role in the stabilization of the secondary and tertiary structure of tRNA and studies have indicated that it stabilizes RNA base stacking and codon–anticodon interactions (Davis and Poulter 1991; Davis 1995; Davis et al. 1998). In addition, Ψ modifications in snRNAs have been shown to be necessary for pre-mRNA splicing in a *Xenopus* oocyte splicing system (Yu et al. 1998; Zhao and Yu 2004)

and in an *in vitro* reconstitution/complementation assay using HeLa cell extracts (Donmez et al. 2004). The presence of Ψ has also been shown to play a critical role in the regulation of transcription by nuclear receptors (Zhao et al. 2004b, 2007).

This modification is formed by a group of enzymes known as pseudouridine synthases, of which there are five families: RluA, RsuA, TruA, TruB, and TruD (Hamma and Ferré-D'Amaré 2006). While there is significant homology among Ψ synthases in a family, homology between the various families is relatively low (Koonin 1996; Hamma and Ferré-D'Amaré 2006). Of the eukaryotic Ψ synthases, the TruA family has been studied most extensively, and human Pseudouridine synthase 1 (hPus1p) is a member of this family. *Saccharomyces cerevisiae* Pus1p was shown to modify uridines at 1, 26, 27, 28, 34, and 36 *in vitro*, and 65 and 67 *in vivo*, as well as position 43 in U2 snRNA *in vitro* and *in vivo* (Motorin et al. 1998; Massenet et al. 1999). Mouse Pus1p (mPus1p) has been shown to modify positions 27, 28, 34, and 36 *in vitro* (Chen and Patton 1999). Additionally, positions 1 and 30 on tRNA and position 43 on *S. cerevisiae* U2 snRNA are modified when mPus1p was

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expressed in a Pus1p-deficient *S. cerevisiae* strain (Behm-Ansmant et al. 2006). When the *pus1* gene was knocked out in *Caenorhabditis elegans* there was no effect on the modification of U2 snRNA isolated from worms, but the modification of tRNAs at Pus1p-dependent positions 27 and 28 was lost (Patton and Padgett 2003).

From the crystal structure of bacterial TruA it is known that the active site of this original member of the TruA family is populated by four strictly conserved amino acids (Foster et al. 2000). These include a catalytic aspartate (D60 in TruA), two arginines (R58 and R205), and a tyrosine (Y118). These residues correspond to D118, R116, R267, and Y173 in hPus1p (see Fig. 1). In addition to the conservation of these four amino acids among the TruA Ψ synthases, there is a particularly high level of sequence homology in the areas immediately surrounding these residues (see Fig. 1). Previous mutation studies on Ψ synthases have shown that the catalytic aspartate is absolutely critical for the enzymatic activity and cannot be replaced with any other amino acid (Huang et al. 1998; Conrad et al. 1999; Ramamurthy et al. 1999; Raychaudhuri

et al. 1999; Zebardjian et al. 1999; Del Campo et al. 2001; Kaya and Ofengand 2003; Zhao et al. 2004b). Analysis of the TruA crystal structure in addition to structural studies of members of other Ψ families has suggested that the two arginines (R58 and R205 in TruA) play a role in constraining the aspartate and in flipping out the uracil base of the substrate allowing it to be modified (Foster et al. 2000; Hamma and Ferré-D'Amaré 2006; Hur et al. 2006; Hur and Stroud 2007). The role of the tyrosine at 118 in TruA has not been fully elucidated; however, a previous mutation study using TruB Ψ synthase has shown that replacement of the tyrosine with alanine, phenylalanine, or leucine results in the lack of enzymatic activity on a natural substrate (Phannachet et al. 2005).

In humans, a single nucleotide mutation in the *PUS1* gene results in the change of the arginine at 116 to tryptophan and has been correlated with the disease mitochondrial myopathy and sideroblastic anemia (MLASA) (Bykhovskaya et al. 2004). Extracts from patient derived cell lines show no hPus1p activity in vitro, and tRNAs from those cell lines lack Ψ at positions in

human Pus1p	-----	--MAGNAEPP	PAGAACPDQR	RSCSGRAGGD	RWEDGGEHPA	KKLKSGGDEE	R-----	REKF	53	
mouse Pus1p	-----	--MAGN-KVP	PALASHQPD	K---GRGGW-	LVWETEEHPA	KRVKSGEDEE	P-----	PRKL	47	
C. elegans Pus1p	-----	-----	-----	-----MTK	TVETOTVDKL	NHVQSGGVHK	E-----	PKKE	28	
yeast Pus1p	MSEENLRPAY	DDQVNEDVYK	RGAQSKLTKA	RKADFDEK	KKKNDKMLD	KRFKSGPRLD	ENGNNLPKRP		70	
E. Coli truA	-----	-----	-----	-----	-----	-----	-----	MSDQQ	5	
human Pus1p	--PKRKIVLL	MAYSCKGVYHG	MORNVGSSQF	RTIEDDLVSA	LVRSGCIPEN	HGEDMRKMSF		QRCARTDKGV	121	
mouse Pus1p	--PKRKIVLL	MAYSCKGVYHG	MORNVGSSQF	RTIEDDLVSA	LVRSGCIPEN	HGEDMRKMSF		QRCARTDKGV	115	
C. elegans Pus1p	RVKQQRHML	LVYQGRKYHG	MLQ---KDL	FTIBSKLLBA	METAGWTTA	QKEKPFDFEF		QRAARTDRAV	95	
yeast Pus1p	RLPKRKIVVM	VGYCSTGYHG	MOYN---PPN	FTIBSAIFKA	FTWAGATSKD	NSNDLKKNGF		MRAARTDKGV	137	
E. Coli truA	QFFVVKIALG	IEYDSSKMYG	WQRQ---NEV	RSVQEKLEKA	LSQVA-----	---NEPITV		FCAGRTDAGV	63	
human Pus1p	SAAGQVVSLL	VWLIDDILEK	-----INSHL	FSHIRILSLK	RVTGGFNSKN	KCDARTYQYL		LPTFAF----	182	
mouse Pus1p	SAAGQVVSLL	VWLIDDILDK	-----INSHL	FSHIRILSLK	RVTGGFNSKN	KCDARTYQYM		LPTFAF----	176	
C. elegans Pus1p	SAARCMGMO	LPRDDAKYQA	EGAGILNNIL	PADIRVCGMR	RTTNFHFPOK	QCDHRTYSMT		CESEFV----	161	
yeast Pus1p	FAENLLISLK	MLIEDPDIQK	K---INERKL	REGIRVWDIE	RVNKAFCRCK	MCSSRWYDYL		LPTVSLIGPK	203	
E. Coli truA	HGTQGVVHFE	TTAQRKDAAM	TLG--VNANL	EGDIAVRVWK	AMPDDPHARF	SATARRRYMI		TYNHRL----	127	
human Pus1p	-----	-----	-----	-----	-----	-----	-----	AHKDR	IVQDE-----	192
mouse Pus1p	-----	-----	-----	-----	-----	-----	-----	AHKDR	IVQDE-----	186
C. elegans Pus1p	-----	-----	-----	-----	-----	-----	-----	A-KPT	ELTNS-----	170
yeast Pus1p	PGSILYRDIE	ESKTELPGLV	DEDLESKEFW	EEFKKDANEK	FSTEEIEAIL	AYVPPARDEF		DINEELYQKV		273
E. Coli truA	-----	-----	-----	-----	-----	-----	-----	RPA	VLSKGV-----	136
human Pus1p	-----	--TYRLSAET	LQQVNELLAC	YKGTNHFHNF	TSQKGPQIPS	ACRYILEMYC		EEPFV----		245
mouse Pus1p	-----	--SYRLSAET	LQQVNELLAC	YKGTNHFHNF	TSQKGFREPS	ARRYILEMYC		EEPFV----		239
C. elegans Pus1p	-----	--SFRLSEET	LAVNSILSI	YLGTHNFHNF	TAKRAYDDMS	SNRYTISFEC		KEPFLFRDDF		228
yeast Pus1p	KKYKQLENAH	RRRYRISAAK	LAKFRASTSQ	YLGTHNFHNF	TLGKDFKHS	AIRFMKDIKV		SDPFVIGD--		341
E. Coli truA	-----	--THFYEPID	AERMHEAQQ	ILGNDFTST	RAVQC-QSRT	PWNVVMHNV		T-----		184
human Pus1p	-REGIEPAVI	RVKGQSFMMH	QIRKMVGLV	AIKGYAPES	VLERSWGTEK	VDVPAKPLG		LVLERVHFKE		314
mouse Pus1p	-REGIEPAVI	RVKGQSFMMH	QIRKMVGLV	AIKGYAPES	VLERSWGTEK	VDVPAKPLG		LVLERVHFKE		308
C. elegans Pus1p	RKEDVEFQI	VTKGQSFVLH	QIRKMVGMVI	TVIRELHLKS	STQSFEGQR	MDIPKAPGLG		LLLERVHFYS		240
yeast Pus1p	--AQTEWISI	KTEGQSFMMH	QIRKMVEMAT	LITRCGQVE	RISQAYGQCK	INIFKAPALG		LLLEAPVFE		409
E. Coli truA	--RHGPYVVV	DIKANAEVHH	MVNINIGSLM	EVGAHNQES	WIAELLAARD	RTLAARATAK		EGYLVAVDY		252
human Pus1p	YNCRFGNDGL	HEPLDWAQE	-EGKVAAFE	EHYPTIIGT	ERDRSMACW	LSTLPIHNS		ATALTAGG--		380
mouse Pus1p	YNCRFGNDGL	HEPLDWTQE	-EGKVTAFFE	QYIYPTIVST	ERDRSMACW	LNTLPIHNS		GTALGAAD--		374
C. elegans Pus1p	YDRK--NSKS	HEPLDWDGEI	IEKEIERTKF	ELITKDMIET	ELLTQGMKWK	LAILVHHPT		ANFAEPEPK		308
yeast Pus1p	YNKRL--EQF	GKALDFSKY	-QDEVKFKM	KHITDKYKE	EVDENVNFAF	FSYIDSFNKK		TGAQGEETAD		476
E. Coli truA	PDRYDLKPP	MGFLPLAD	-----	-----	-----	-----		-----		270
human Pus1p	---TGAKVP	SPLEGSEGDG	DTD	-----	-----	-----		-----		399
mouse Pus1p	---TGAKVP	SSLEGSEGDG	DTD	-----	-----	-----		-----		393
C. elegans Pus1p	KTFVIMAAAC	ASKAAKEGEG	VAPEAQDAPK	EAEAPAEVTP	EPVAATSSG	-----		-----		358
yeast Pus1p	KSGPAVQKSI	FEFLTAKSIP	GLTDAPESNK	KIKQRKRMEE	EAAASKKAEI	SSTTQSENEP		VQPEAAAN		544
E. Coli truA	-----	-----	-----	-----	-----	-----		-----		-----

FIGURE 1. The amino acid sequences of human, mouse, *C. elegans*, and *S. cerevisiae* Pus1p, as well as *E. coli* TruA are aligned to show homology. Positions where at least three of the five proteins show identity have been highlighted.

tRNAs expected to be modified by the hPus1p enzyme, even though the mutant protein is still present (Patton et al. 2005). Since this is not the position of the critical catalytic aspartate (D118) it has been suggested that the nonconservative replacement found in the patients, R116W, sterically hinders the active site and results in an inactive enzyme.

We hypothesized that any substitution at these highly conserved sites in hPus1p (116, 173, and 267) would affect the function of the synthase and that anything other than conservative replacement would not be tolerated. To test this we created a number of single amino acid replacement mutants at these positions using both conservative and nonconservative substitution. Surprisingly, many of the mutants retained partial Ψ synthase activity at positions in tRNA known to be modified by Pus1p, and these mutants exhibit differential activities at particular positions on tRNA substrates instead of exhibiting a general reduction of activity.

RESULTS

The wild-type hPus1p used in these studies (see Fig. 1) is predicted from the DNA sequence to have an additional 51 amino acids on the amino terminus compared with the partial human sequence published previously (Chen and Patton 1999). A partial human protein, starting with the methionine at position 62, which is in the middle of a region conserved in TruA synthases (see Fig. 1), was expressed in bacteria and the isolated protein had no activity (Chen and Patton 1999). The wild-type hPus1p used in this study, with the additional amino acids, starting with position 1 in Figure 1, containing a histidine leader at the amino terminus, was expressed in bacteria, isolated, and tested in Ψ synthase assays (see below).

In order to elucidate the role of active site amino acids R116, R267, and Y173 in hPus1p, we used site-directed mutagenesis to create mutations at these positions. The mutations included both conservative and nonconservative replacements at each site. Characteristics of Pus1p include its specificity for the same positions on a wide variety of tRNAs and its multisite specificity (Motorin et al. 1998; Chen and Patton 1999; Behm-Ansmant et al. 2006). For this reason, we chose to test the mutants on multiple tRNA substrates. All of the substrates were generated using in vitro transcrip-

tion as described in the methods, and the sequences of all of the substrates used can be found in Figure 2.

The assay used as a first screen of wild-type and mutant activities is the tritium release assay, which provides a quantitative measure of total Ψ formation following incubation of the enzyme with uridine 5- 3 H-labeled substrate. It is a simple assay that allows for the testing of the numerous mutant enzymes and several tRNA substrates in order to quantitate total activity (Table 1). The values in the table indicate the number of moles Ψ produced per mole of tRNA. One mole Ψ /mole tRNA is expected for each predicted position; however, some positions may not be completely modified in vitro, resulting in a lower than expected number even for wild-type hPus1p. The number of possible positions for each substrate can be found at the top of the columns, and most have already been shown to be modified by mPus1p in vitro (Chen and Patton 1999; Behm-Ansmant et al. 2006). Since the human enzyme is highly homologous to mPus1p (90% identity, 4% conservative replacement) (see Fig. 1), it was expected hPus1p would also modify all the same substrates at the same positions.

Since mPus1p is well characterized (Chen and Patton 1999, 2000; Behm-Ansmant et al. 2006) and this is the first test of hPus1p in vitro, the activities for mPus1p and hPus1p were compared. The two Ψ synthases had very similar activities

FIGURE 2. Structures of tRNA substrates used to test the activity of wild-type and mutant hPus1ps. The Ψ residues that are modified by hPus1p are surrounded by a rectangle and the position denoted to the left. Human cytoplasmic tRNA^{Ser}_{UGA} (A), mouse cytoplasmic pre-tRNA^{Ile}_{UAU} (B), human cytoplasmic tRNA^{Ile}_{AAU} (C), and mouse cytoplasmic tRNA^{Met}_{CAU} (D) are shown with predicted secondary structures. In the *S. cerevisiae* pre-tRNA^{Ile} (B,E), the arrows define the intron of the pre-tRNA.

TABLE 1. ^3H -release assays of mPus1p, hPus1p, and position 116 mutants with various substrates

Enzyme	<i>S. cerevisiae</i> pre-tRNA ^{Ile} (Ψ at 27, 30, 34, 36) ^a	Mouse tRNA ^{Met} (Ψ at 27) ^b	Mouse pre-tRNA ^{Ile} _{UAU} (Ψ at 30)	Human tRNA ^{Ile} _{AAU} (Ψ at 27, 30)
mPus1p	3.12 $\pm$ 0.67 ($n = 2$)	0.91	N.D.	N.D.
hPus1p	3.12 $\pm$ 0.63 ($n = 4$)	0.98	0.77	0.92 $\pm$ 0.03 ($n = 2$)
R116A	0.46 $\pm$ 0.25 ($n = 2$)	0.09	<0.01	0.74
R116C	1.18 $\pm$ 0.03 ($n = 2$)	N.D.	N.D.	0.74 $\pm$ 0.04 ($n = 2$)
R116E	0.00	N.D.	N.D.	N.D.
R116G	0.44	N.D.	<0.01	0.67
R116H	0.06 $\pm$ 0.04 ($n = 2$)	0.01	N.D.	N.D.
R116K	1.33 $\pm$ 0.15 ($n = 3$)	0.76	0.02	0.80 $\pm$ 0.01 ($n = 2$)
R116N	0.46	N.D.	0.01	0.72
R116Q	0.20	N.D.	N.D.	N.D.
R116S	0.59	N.D.	0.01	0.73
R116W	0.03 $\pm$ 0.02 ($n = 3$)	0.01	N.D.	N.D.

Activity is reported in mol Ψ /mol tRNA.

^aThe positions of Ψ that are possible Pus1p modification sites are given under the substrate in the column heading. The activity is in mol Ψ /mol tRNA and has had the value measured for incubation of the substrate for the same length of time with bacterially expressed Lac subtracted. A value of "0.00" means there was no activity above background in this sample. With yeast pre-tRNA^{Ile} and human tRNA^{Ile}_{AAU}, n denotes the number of determinations on separate days and, when n is not given, it is equal to 1. With mouse tRNA^{Met} and pre-tRNA^{Ile}_{UAU} the values reported are the means of three assays of the same reaction and all of the SDs were <0.03 for tRNA^{Met} and 0.01 for mouse pre-tRNA^{Ile}_{UAU}.

^bN.D. denotes "not determined."

(Table 1) on *S. cerevisiae* pre-tRNA^{Ile} (γ pre-tRNA^{Ile}) and mouse tRNA^{Met} (m tRNA^{Met}). Each modified the γ pre-tRNA^{Ile} at slightly higher than 3 mol of Ψ /mol tRNA, denoting that at least three uridines have been converted to Ψ . With the m tRNA^{Met} substrate, again the activities were quite similar, with ~ 1 mol Ψ /mol tRNA for each of the two enzymes, the expected level of Ψ with this substrate.

Next we tested hPus1p mutants at the R116 position, the residue that is mutated to tryptophan in some patients with MLASA (Bykhovskaya et al. 2004). The mutants can be divided into three groups based upon their activity with γ pre-tRNA^{Ile}. R116K and R116C retained the most activity with approximately one-third that of wild-type levels. The replacement of arginine with lysine is a conservative one, retaining the charge and approximate size of the arginine side chain. The side chain on cysteine is not charged at neutral pH and its size is considerably smaller than that of arginine or lysine.

The second group, composed of the R116A, R116G, R116N, R116Q, and R116S mutants, all exhibited some activity with the γ pre-tRNA^{Ile} substrate (Table 1). All of the side chains for these amino acids are relatively small and are either nonpolar or polar and uncharged at neutral pH. The R116E, R116H, and R116W (the mutation in MLASA patients) mutants make up the third group and were essentially without activity. Tryptophan has a large bulky side chain and glutamic acid's side chain is negatively charged, replacing the positively charged side chain of arginine. Although the positive charge is retained with

the R116H mutation, it is much more constrained than the side chain for arginine.

With the m tRNA^{Met} substrate, where only one position (27) (see Fig. 2) is expected to be modified by hPus1p, the results are very interesting. The wild-type mPus1p and hPus1p show the formation of nearly 1 mol Ψ /mol tRNA as reported above, but the R116K mutant exhibited an activity of 0.76 mol Ψ /mol tRNA for this substrate, a level of activity that approaches that of wild type. The other mutants tested with the m tRNA^{Met} substrate had either low levels of activity, as with R116A, or no activity, as with R116H and R116W.

All of the mutants that showed some activity with the γ pre-tRNA^{Ile} substrate were tested on human tRNA^{Ile}_{AAU} (h tRNA^{Ile}_{AAU}). This tRNA has two possible uridines that could be modified by hPus1p, positions 27 and 30. Wild-type hPus1p only shows activity at the level of 0.92 mol Ψ /mol tRNA (Table 1).

This suggests that approximately one site is being modified by hPus1p, most likely position 27. Nonetheless, all the mutants that were tested had significant activity (between 0.67 and 0.80 mol Ψ /mol tRNA) on this substrate, a result reminiscent of the activity of R116K on m tRNA^{Met}; but with h tRNA^{Ile}_{AAU}, even R116A, C, G, N, and S have significant levels of activity, compared with wild-type hPus1p.

When mouse pre-tRNA^{Ile}_{UAU} (m pre-tRNA^{Ile}_{UAU}) (see Fig. 1) is used as a substrate, where only position 30 can be modified by hPus1p, only the wild-type enzyme exhibits any appreciable activity. There is a clear dichotomy between wild-type hPus1p and all the mutants tested, with wild-type hPus1p exhibiting 0.77 mol Ψ /mol tRNA, and R116K, the mutant with the highest activity, is limited to 0.02 mol Ψ /mol tRNA.

Tritium release assays were also performed with mutants at the Y173 and R267 positions to determine if any activity would be retained when these strictly conserved amino acids were replaced. Table 2 contains the tritium release data for the Y173 and R267 mutants incubated with the γ pre-tRNA^{Ile} substrate, conducted in separate experiments from those presented in Table 1. The Y173C, Y173F, Y173G, and Y173T mutants all retained some activity; however, it was relatively low (0.54 mol Ψ /mol tRNA at best) (Table 2). The combination of hydroxyl group and hydrophobic ring appears to be required for activity since Y173F, Y173T, and Y173S are minimally active or totally inactive.

TABLE 2. ^3H -release assays of hPus1p and position 173 and 267 mutants with *S. cerevisiae* pre-tRNA^{lle}_{UAU}

Enzyme	<i>S. cerevisiae</i> pre-tRNA ^{lle} _{UAU} (Ψ at 27, 30, 34, 36) ^a
hPus1p	2.94 $\pm$ 0.48. (<i>n</i> = 3)
Y173C	0.52 $\pm$ 0.06. (<i>n</i> = 2)
Y173F	0.25 $\pm$ 0.08. (<i>n</i> = 2)
Y173G	0.29 $\pm$ 0.04. (<i>n</i> = 2)
Y173S	0.01 $\pm$ 0.00. (<i>n</i> = 2)
Y173T	0.54 $\pm$ 0.07. (<i>n</i> = 2)
R267G	0.09
R267K	1.12

Activity is reported in mol Ψ /mol tRNA.

^aThe positions of Ψ that are possible Pus1p modification sites are given under the substrate in the column heading. The activity in mol Ψ /mol tRNA has had the value measured for incubation of the substrate for the same length of time with bacterially expressed Lac subtracted. The number of determinations on separate days used to obtain the mean is stated in parentheses. With the R267 mutants the values reported are the means of three assays of the same reaction and the SDs for R267G and R267K are <0.01 and 0.03, respectively.

From the structure of TruA, the arginines at 116 and 267 might be considered equivalent positions and the R267K mutant retained approximately one-third of wild-type activity with the γ pre-tRNA^{lle} substrate (Table 2), the same level observed with R116K. The same correspondence is not true for the R267G mutant, which exhibited negligible activity with this substrate (0.09 mol Ψ /mol tRNA) (Table 2), considerably less than that of R116G (0.44 mol Ψ /mol tRNA) (Table 1).

Since the data in Table 1 suggested that certain mutants might modify position 27, the R116K and R267K mutants were chosen for further analysis employing substrates that can be modified by hPus1p at only one residue, position 27 (m tRNA^{Met}) or position 28 (h tRNA^{Ser}). Wild-type hPus1p and the R116K mutant were incubated with m tRNA^{Met}; time points were taken at 15, 30, 60, and 120 min, and the levels of activity were measured using the tritium release assay. Wild-type hPus1p and the R267K mutant were incubated with h tRNA^{Ser} for 30, 60, and 120 min. As can be seen in Figure 3A, the R116K mutant showed a near wild-type rate of Ψ formation and final level of pseudouridylation. The R267K rate and total activity on h tRNA^{Ser} was indistinguishable from that of wild-type hPus1p (Fig. 3B). This indicates that, at least for positions 27 and 28 in long-term incubations, the activity of these mutant enzymes is nearly equal to that of wild-type hPus1p.

However, short-term kinetic analysis (1- and 2-min incubations) (see Materials and Methods) of wild-type hPus1p and the R116K mutant using the γ pre-tRNA^{lle} substrate yielded apparent K_m values of 32 ($\pm$ 1) nM for wild-type hPus1p and 168 ($\pm$ 20) nM for the R116K mutant. The $V_{\max}$ values determined were 54 ($\pm$ 4) and 56 ($\pm$ 8) fmol/min for wild-type and the R116K mutant,

respectively. The equilibrium binding constants obtained with the same substrate are 250 nM for wild-type hPus1p and 420 nM for R116K.

In order to determine whether the mutant enzymes were preferentially modifying certain positions on tRNA substrates that have more than one position that can be modified, or if all of the possible positions were being incompletely modified, an assay other than ^3H -release was required. The CMCT Ψ -mapping assay, where there is a reverse transcription stop one position before a covalently modified Ψ , was used to precisely determine which uridine was modified on the γ pre-tRNA^{lle} substrate after incubation with R116C, R267K, and wild-type hPus1p.

The presence of Ψ is indicated by more intense bands in the CMCT lanes than in the control lane (–CMCT) one position prior to a U. Arrows in Figure 4 denote the positions beneath the predicted Ψ s where the stops should be observed. In Figure 4A we show that wild-type hPus1p creates Ψ at all predicted positions: 27, 30, 34, and 36 (Fig. 4A, lanes 1–3). The level of Ψ at position 36, even with wild-type hPus1p, is considerably less than that of the other positions (27, 30, and 34), which agrees with earlier characterization of mPus1p (Behm-Ansmant et al. 2006). The CMCT result is consistent with the tritium release assay data where a value of $\sim$ 3.00 mol Ψ /mol tRNA was

FIGURE 3. Time course of wild-type and mutant hPus1p activity. Reactions were incubated for the times shown and the levels of Ψ formed were determined by ^3H -release assays (see Materials and Methods). For the m tRNA^{Met} substrate (A, wt hPus1p and the R116K mutant) the SDs for the three assays of the same reaction were all $\leq$ 0.04 mol Ψ /mol tRNA. With the h tRNA^{Ser} substrate (B, wt hPus1p and the R267K mutant) only a single assay was carried out for each time point.

FIGURE 4. CMCT-primer extension assay of activity for wild-type and mutant hPus1p on *S. cerevisiae* pre-tRNA^{Ile}. These assays were carried out on in vitro modified *S. cerevisiae* pre-tRNA^{Ile}(UAU) incubated for three hours with either wild-type (wt), mutant (R116C [A] or R267K [B]) hPus1p, or with Lac protein as described in Materials and Methods. The filled triangles denote an increase in CMCT concentration from 0.042 to 0.167 M CMCT in the chemical modification portion of the assay and a “–” denotes that no CMCT was included. Sequence lanes are deduced from chain terminating reactions using the same primer used in the CMCT assays, with the *S. cerevisiae* pre-tRNA^{Ile}_{UAU} plasmid as the template. The uridines converted to Ψ are noted to the right of each panel and the positions of the stops to reverse transcriptase just prior to those uridines are denoted on the left of each panel.

obtained for wild-type hPus1p with this substrate (see Tables 1, 2). Interestingly, a Ψ is indicated strongly at position 27 when the R116C mutant was used for modification, but the bands at the other positions (30 and 34) are not as intense as the bands in the wild-type hPus1p lanes (Fig. 4A, cf. lanes 2,3 and 5,6). This result is consistent with the value of 1.18 mol Ψ/mol tRNA in the ³H-release data (Table 1) for this substrate with R116C.

In Figure 4B, a comparison of wild-type hPus1p and the R267K mutant shows the same result. There is a prominent band at position 27 (CMCT treated) in both the wild-type and R267K incubated lanes, and no band in the untreated lanes (Fig. 4B, cf. lanes 4–6 and 7–9). At position 30, with wild-type hPus1p, there is a band of increasing intensity in the CMCT-treated lanes (Fig. 4B, lanes 4–6), but with R267K there is little or no increase in the intensity of the band with CMCT treatment (Fig. 4B, lanes 7–9), indicating there is little, if any, Ψ formed at this position. With wild-type hPus1p there is an increase in the intensity of the band at position 34 but there is no increase with the R267K mutant at this position. At position 36 the relatively high background coupled with the fact that the level of pseudouridylation at this position is low even with wild-type hPus1p precludes assaying the level of modification at this position. Nonetheless, it is obvious from the two panels that, although the mutants can modify the uridine at position 27, the other positions are left largely unmodified when compared with wild-type hPus1p.

The CMCT assay is only semi-quantitative and we wanted to measure the levels of Ψ at all the positions modified by hPus1p in γ pre-tRNA^{Ile}. A 2D-TLC procedure

was used to obtain a quantitative value for the amount of Ψ formed at position 27, position 30, and positions 34 and 36 (combined) in γ pre-tRNA^{Ile} when hPus1p, a Lac control, and the R116A, R116K, R116S, Y173C, and R267K mutants were used in the modification reactions. The strategy for this method involves labeling the tRNA substrate used in the reactions with one of three α-³²P-labeled NTPs. When the tRNA is digested with RNaseT₂ after incubation with the Ψ synthases, the nucleotide 5' to the labeled nucleotide will retain the labeled phosphate, effectively transferring the label and yielding a 3'-labeled nucleotide that can be chromatographed on thin-layer plates. By judicious selection of the labeled NTP used during the synthesis of the γ pre-tRNA^{Ile}, we can determine the levels of Ψ at the positions modified by hPus1p. Figure 5A contains the images from the assay to determine Ψ at position 27.

The five spots on the wild-type hPus1p panel represent the four nucleotides (NMPs, with a 3' phosphate) and Ψp on the tRNA that occur 5' to a cytosine. A Ψ spot is visible in every image except for Lac, indicating that all of the mutants and wild-type hPus1p formed Ψ at position 27.

The spots in Figure 5B represent Ψs 5' to an adenine; this includes positions 34 and 36, but will measure the level primarily at position 34 given the known activity of mPus1p (Behm-Ansmant et al. 2006) and what was shown for hPus1p in Figure 4. A Ψ spot is clearly visible in only the chromatogram where wild-type hPus1p was incubated with the substrate. This indicates that none of the mutants tested in this assay retained significant activity at positions 34 or 36 and is consistent with what was shown earlier using the CMCT assay (Fig. 4). Figure 5C contains the images for determining the level of Ψ at position 30. A Ψ spot is visible on these overexposed TLCs when both wild-type hPus1p and the R267K mutant were used, though the Ψ spot is much more apparent on the wild-type hPus1p chromatogram.

The intensity of the spots in the chromatograms shown in Figure 5 was measured using a phosphorimager. These data are presented as mol Ψ/mol tRNA and as the percent of wild-type hPus1p activity in Table 3. All of the mutants except Y173C showed near wild-type activity at position 27. None of the mutants showed substantial activity at positions 34 and 36, only R116A and R116K had any activity above zero. All of the mutants registered at least some minimal activity at position 30 with significant, but still low, activity exhibited by the R116K and R267K mutants.

FIGURE 5. Assay of the level of modification at each position in *S. cerevisiae* pre-tRNA^{Ile}_{UAU} by wild-type and mutant hPus1ps. Autoradiographs of portions of each of the two-dimensional TLCs are shown. Substrate tRNA, labeled with the nucleotide noted in the panel, (A) position 27, ³²P-CTP labeled, (B) position 34, ³²P-ATP labeled, and (C) position 30, ³²P-GTP labeled, was incubated with wild-type or mutant hPus1p for three hours, the RNA in the reaction isolated, gel-purified, digested with RNase T₂, and chromatographed in two dimensions on cellulose TLC plates (10 cm×10 cm) as described in Materials and Methods. The position on pre-tRNA^{Ile} assayed is noted in each panel and the spots are identified in at least one chromatogram in each panel.

DISCUSSION

Comparison of TruA and Pus1p

TruA has been the focus of several structural studies and is the founding member of the family that includes Pus1p. These studies (Foster et al. 2000; Hur and Stroud 2007) provide a framework to discuss the present results, given the structure of Pus1p has not been solved from any species. There are some notable differences that have been observed for TruA and Pus1p even though they are members of the same family. TruA is a homodimer in its tRNA-binding, active form (Foster et al. 2000), whereas *S. cerevisiae* Pus1p is not (Arluison et al. 1999a). Although TruA requires the entire tRNA as a substrate for modification (Hur et al. 2006), *S. cerevisiae* Pus1p can modify a mini-substrate composed of an anticodon loop containing an intron (Motorin et al. 1998). TruA modifies only uridines found at positions 38, 39, and 40 in a number of *Escherichia coli* tRNAs, but Pus1p modifies uridines at positions 1, 26, 27, 28, 30, 34, 36, 65, and 67 depending on

the source of the enzyme (*S. cerevisiae* or mouse), the substrates (intron-containing or not), or whether the activity was monitored in vivo or in vitro (Simos et al. 1996; Motorin et al. 1998; Chen and Patton 1999; Behm-Ansmant et al. 2006). *S. cerevisiae* Pus1p has been shown to modify uridines at all the positions stated except position 30 (Motorin et al. 1998; Behm-Ansmant et al. 2006), and mouse Pus1p has been shown to modify uridines at positions 1, 27, 28, 30, 34, and 36 (Chen and Patton 1999; Behm-Ansmant et al. 2006). Out of all the positions that Pus1p modifies, the modification of uridines at positions 27 and 28 is by far the most common in tRNAs, with Ψ infrequently found at the other positions that the Pus1p enzymes recognize (Sprinzl et al. 1998). Even with these differences between Pus1p and TruA, the amino acids composing the active sites of these enzymes are highly conserved and the general mechanisms of substrate recognition and modification are probably comparable since all Ψ synthases, in all families that have been identified thus far, have a core structure (for review, see Hur et al. 2006).

TABLE 3. Quantitation from 2D TLCs of *S. cerevisiae* pre-tRNA^{lle}_{UAU} labeled with $\alpha^{32}\text{P}$ -NTP incubated with wild-type (wt) hPus1p and various mutants

Enzyme	CTP labeled (Ψ at 27) ^a		ATP labeled (Ψ at 34, 36)		GTP labeled (Ψ at 30)	
	$\frac{\text{mol } \Psi}{\text{mol tRNA}}$	Percent of wt (%)	$\frac{\text{mol } \Psi}{\text{mol tRNA}}$	Percent of wt (%)	$\frac{\text{mol } \Psi}{\text{mol tRNA}}$	Percent of wt (%)
wt hPus1p	0.76	100	0.92	100	0.45	100
R116A	0.68	89	0.06	8	0.03	7
R116K	0.75	99	0.08	9	0.05	11
R116S	0.79	104	0.00	0	0.02	4
Y173C	0.38	50	0.00	0	0.01	2
R267K	0.71	93	0.00	0	0.10	22

Activity is reported in mol Ψ /mol tRNA and as the percentage of wt hPus1p at that position.

^aThe positions of Ψ that are possible Pus1p modification sites given the nucleotide label are given next to the labeled nucleotide in the column heading. The counts were measured on the TLC plates using a PhosphorImager. The activity in mol Ψ /mol tRNA has had the value measured for incubation of the substrate for the same length of time with bacterially expressed Lac subtracted. A value of "0.00" indicates that there was no activity above background observed.

Arginines and tyrosine in the active site

Foster et al. (2000) showed that the arginines at positions 58 and 205 in TruA (equivalent to 116 and 267 in hPus1p) interact with the active site aspartate (D60 in TruA, D118 in hPus1p) through bridging water molecules. Mutation of R58 to alanine in TruA results in an inactive enzyme that still binds tRNA with a similar affinity as wild-type TruA (Hur and Stroud 2007) and led these investigators to conclude that R58 facilitates base-flipping rather than taking part in the recognition or catalysis steps (Hur and Stroud 2007). The equivalent position (R62) in RluA also appears to have a key role in the flipping of the target base (Hoang et al. 2006) and, when R62 was replaced with either lysine or methionine, all activity was lost in RluA (Hoang et al. 2006). Given that patients with MLASA have a mutation at the equivalent position in hPus1p (R116W) that also renders the enzyme inactive (Bykhovskaya et al. 2004; Patton et al. 2005), one would assume that there would be little tolerance for substitution at this position. Our data show that in fact several of the hPus1p mutants at position 116 exhibit significant activity, especially when the modification at positions 27 or 28 is considered. The levels of modification at additional sites such as 30 and 34 are greatly diminished, but that "core" activity at 27/28 is retained, albeit with an increased K_m and slightly reduced affinity for the substrate. The same is true for mutations at other positions (Y173 and R267) in hPus1p; some mutations result in no activity, such as Y173S, but others show some activity toward modification at position 27 in the *S. cerevisiae* pre-tRNA^{lle}. When the corresponding tyrosine in *E. coli* TruB (Y67) was mutated to phenylalanine, leucine, or alanine, there was no detectable activity on a natural tRNA substrate (Phannachet et al. 2005), suggesting that there was a need for both a hydrophobic ring and an OH group combined in one side chain (Phannachet et al. 2005). These data would suggest that the functions of these highly conserved amino acids are less constrained in hPus1p than

for instance R62 in RluA, R58 in TruA, and Y67 in TruB. The substitution of R116 with glutamic acid, histidine, or tryptophan is not tolerated in hPus1p because of either steric or charge constraints, but there are not strict requirements for the presence of arginine at this position in hPus1p, at least when the modification of position 27/28 is considered.

If R116 and R267 were essential to the catalytic function of hPus1p we would expect to see an approximately equal decrease in activity at all positions on the substrate. However, all of the mutants retained activity almost exclusively at position 27 or 28. This confirms that the role of the two arginines is more likely one of substrate manipulation. As mentioned previously, by comparison with TruA, the putative role of R116 in hPus1p is to flip the uridine out of the RNA and into the active site of the enzyme and at the same time stabilize the RNA structure by substituting for the flipped out uridine (Hur and Stroud 2007). We would not expect most of the smaller side chain amino acid mutations, especially glycine, to be able to help fulfill either of these roles. It is possible that the uridines at positions 27 and 28 are especially prone to flipping out of the RNA. Perhaps, since they are at the end of the stem, they are not as stably held as others in the middle of a stem might be, for instance a uridine at position 30.

One could reasonably expect to retain some enzymatic activity when replacing an arginine with lysine, because the two molecules are very similar in size, shape, and charge. This explains why both R116K and R267K were among the mutants with the highest levels of retained activity. They might participate weakly in base flipping and also replace the displaced uridine. The polar side chain of cysteine is much smaller than lysine, has no charge at neutral pH, and has a different functional group (SH), so it is not immediately apparent why the R116C mutant was able to retain activity levels equivalent to R116K. Mutants of R116 with other smaller amino acid side chains such as alanine, glycine, asparagine, and serine all retained some activity,

presumably because, while they might not actively participate in flipping out or replacement of the uridine, neither will they sterically block the active site, as R116W might.

In terms of Y173, it has been suggested that, at least in TruB, the corresponding tyrosine (Y67 in *E. coli* TruB) is involved in maintaining the catalytic aspartate in the charged state through the maintenance of a salt bridge (Hoang and Ferré-D'Amaré 2001; Chaudhuri et al. 2004). Our results with mutations at this position suggest that a tyrosine at this position is indeed critical, but there was detectable activity with all the mutants except Y173S. The two mutants with the highest activity are Y173C and Y173T, both with side chains that could participate in the interaction with the aspartate in the active site, although cysteine has an ionizable side (pK 8.3) chain like tyrosine (pK 10), whereas threonine does not. Why there is detectable activity with the Y173G and Y173F mutants, whose side chains would not be expected to participate in this salt bridge, is unknown. In addition, no activity was seen with Y173S, even though there is an –OH available for interaction with the aspartate. The particular characteristics of the tyrosine side chain, hydrophobicity of the phenyl ring, and an OH group with a pK of 10 appear to be required for the maintenance of the structure of the active site and full activity.

Modification of uridine at position 30

It has been shown that, when mPus1p is expressed in a $\Delta pus1$ yeast strain, position 30 in γ pre-tRNA^{lle}_{UAU} is modified to Ψ . The activity was also observed in vitro with the same substrate and mPus1p, whereas *S. cerevisiae* Pus1p does not exhibit this activity in vivo or in vitro (Behm-Ansmant et al. 2006). In this study we observed that wild-type hPus1p can modify position 30 in γ pre-tRNA^{lle}_{UAU} (Fig. 4) and m tRNA^{lle}_{UAU} (Table 1) in vitro, but probably not at position 30 in h pre-tRNA^{lle}_{AAU}. However, modification of uridine at this position is severely hampered by mutation at any of the three active site residues.

Milder phenotypes anticipated with some mutations and possible recovery of activity

Since the positions that were chosen for mutation are highly conserved in Ψ synthases, it was somewhat surprising to find that any substitution is tolerated. In fact some substitutions at these residues resulted in proteins with nearly a wild-type level of activity at sites 27 and 28 on tRNAs. The R116W mutation seen in MLASA patients may lead to the most severe phenotype and milder phenotypes might occur if a different amino acid was substituted at this position. The codon used to code for arginine at position 116 in humans is CGG and point mutations in the first two positions of the triplet can give rise to five amino acid substitutions, glycine, glutamine, leucine, proline, and tryptophan. The R116G and R116Q mutants had activities

on the level of the R116A mutant and so it is possible that milder phenotypes might result from such mutations.

A recent report showed that enzymatic activity could be recovered in an inactive mutant kinase by the treatment of cells in culture containing the mutant enzyme with small molecules that mimicked the chemistry of the lost amino acid side chain (Qiao et al. 2006). The kinase (protein-tyrosine kinase C-terminal Src kinase) has an active site that pairs an aspartate with a nearby arginine (four residues away) and looks remarkably like the active site of Pus1p. Earlier work had shown that chemical rescue of a mutant enzyme, that replaces the arginine with alanine, was possible and that the class of small molecules that might restore activity could be predicted given knowledge of the composition of the active site (Williams et al. 2000; Zhao et al. 2004a). By understanding the topography of the active site of hPus1p and determining the flexibility of the requirements for particular amino acids that compose it, the pathway to identifying a class of compounds that could be considered for recovery of mutant hPus1p activity might be less uncertain. With the extensive small molecule libraries that are available and the high throughput methods for the screening of these compounds that have been developed, it is now reasonable to assume that a substance might be identified that will restore partial function to the R116W mutant hPus1p or perhaps restore complete activity to other mutants at this or other positions.

MATERIALS AND METHODS

Creation of wild-type and mutant hPus1p

A human *PUS1* cDNA fragment was generated from the I.M.A.G.E. consortium (Lennon et al. 1996) clone #2822709 (obtained from the American Type Culture Collection) using PCR (Mullis et al. 1986) with primers HuPus1pET16bFor (5'-AACATATGGCCGGGAACGCGGAGCC) and HuPus1pET16bRev (5'-GGATCCTCGAGTCCCATCGCCTCAGTCAGTG) as described (Chen and Patton 1999). The PCR fragment was inserted into the pGEMT (Promega) vector and sequenced (Sanger et al. 1977). The fragment was cut from pGEMT with Nde1 and Xho1, gel-purified, and ligated into pET16b plasmid DNA (Novagen) cut with Nde1 and Xho1. The purified hPus1p–pET16b DNA was sequenced, used to transform pLysS (DE3; Promega) cells, and the recombinant hPus1p expressed and isolated as described for mPus1p (Chen and Patton 1999). As with mPus1p, there are an additional 21 amino acids at the amino terminus of hPus1p encoded by the vector (MGHHHHHHHH HHSSGHIEGRH), which are used for purification.

Site-directed mutagenesis was performed with a Quikchange II kit (Stratagene), utilizing the primers listed below, and the hPus1p–pET16b plasmid, to create the hPus1p mutants. The insert was sequenced to confirm the mutation and to make sure no additional mutations occurred. The recombinant mutant proteins were isolated as described (Chen and Patton 1999) except that β -mercaptoethanol was used in the dialysis buffer instead of dithiothreitol; it lowers the amount of precipitation of the protein during dialysis.

Primers 5'-GCGCTGCGCCGCCACAGACAAGGG and 5'-CCC TTGTCTGGCGGCGCAGCGC were used to change R116 to alanine;

Primers 5'-GCGCTGCGCCAACACAGACAAGGG and 5'-CCCT TGTCTGTGTTGGCGCAGCGC to change R116 to asparagine;

Primers 5'-GCGCTGCGCCTGCACAGACAAGGG and 5'-CCC TTGTCTGTGCAGGCGCAGCGC to change R116 to cysteine;

Primers 5'-GCGCTGCGCCGGCACAGACAAGGG and 5'-CCCT TGTCTGTGCCGCGCAGCGC to change R116 to glycine;

Primers 5'-GCGCTGCGCCGAAACAGACAAGGG and 5'-CCCT TGTCTGTTTCGGCGCAGCGC to change R116 to glutamic acid;

Primers 5'-GCGCTGCGCCAGACAGACAAGGG and 5'-CCCT TGTCTGTCTGGGCGCAGCGC to change R116 to glutamine;

Primers 5'-GCGCTGCGCCATACAGACAAGGG and 5'-CCCT TGTCTGTATGGGCGCAGCGC to change R116 to histidine;

Primers 5'-GCGCTGCGCCAAAACAGACAAGGG and 5'-CCCT TGTCTGTTTGGCGCAGCGC were used to change R116 to lysine;

Primers 5'-GCGCTGCGCCAGCACAGACAAGGG and 5'-CCCT TGTCTGTGCTGGCGCAGCGC to change R116 to serine;

Primers 5'-CAGCGCTGCGCCTGGACAGACAAGG and 5'-CCT TGTCTGTCCAGGCGCAGCGCTG were used to change the arginine at 116 to tryptophan;

Primers 5'-GTGATGCCAGGACCTGCTGCTACCTGCTGCC and 5'-GGCAGCAGGTAGCAGCAGGTCTGGCATCAC to change Y173 to cysteine;

Primers 5'-GTGATGCCAGGACCGGCTGCTACCTGCTGCC and 5'-GGCAGCAGGTAGCAGCCGGTCTGGCATCAC to change Y173 to glycine;

Primers 5'-GTGATGCCAGGACCTTCTGCTACCTGCTGCC and 5'-GGCAGCAGGTAGCAGAAGGTCTGGCATCAC to change Y173 to phenylalanine;

Primers 5'-GTGATGCCAGGAACCAGCTGCTACCTGCTGCCC and 5'-GGGCAGCAGGTAGCAGCTGGTCTGGCATCAC to change Y173 to serine;

Primers 5'-GTGATGCCAGGACCACTGCTACCTGCTGCCC and 5'-GGGCAGCAGGTAGCAGGTGGTCTGGCATCAC to change Y173 to threonine;

Primers 5'-GATGCATCAGATCGGCAAGATGGTCGGCCTGG and 5'-CCAGGCCGACCATCTTGCCGATCTGATGCATC were used to convert R267 to glycine; and

Primers 5'-GATGCATCAGATCAAAAAGATGGTCGGCCTGG and 5'-CCAGGCCGACCATCTTTTGTATCTGATGCATC were used to convert R267 to lysine.

tRNA substrate cloning

The tRNA genes for cytoplasmic m pre-tRNA^{lle}_{UAU} and cytoplasmic h tRNA^{lle}_{AAU} were amplified from mouse or human DNA using PCR (Mullis et al. 1986) and the following primers for m pre-tRNA^{lle}_{UAU}: 5'-GGTTGAATTCTAATACGACTCACTATAGCT CCAGTGGCGCAATCGG and 5'-GGTTGGATCCACCATGGT GCTCCAGGTGAGGCTCGAAC; and the following primers for h tRNA^{lle}_{AAU}: 5'-GGTTGAATTCTAATACGACTCACTATAGG CCGGTTAGCTTCAGTTGG and 5'-GGTTGGATCCTGGTGGC CAGTACGGGGATCG. The gene for mouse cytoplasmic tRNA^{Met} was amplified from mouse genomic DNA using the following primers: 5'-GGATGAATTCTAATACGACTCACTATAGCCTCG TTAGCGCAGTAGG and 5'-CCATGGATCCTGGTGCCCCGTG

TGAGGATCG. The resulting fragments were digested with BamH1 and EcoR1, gel-purified, and ligated into gel-purified pUC19 plasmid DNA digested with BamH1 and EcoR1. The resulting constructs have a T7 RNA polymerase promoter at the 5' end of the tRNA gene and a BstN1 site at the 3' end of the gene so that plasmids digested with BstN1 will yield tRNAs with CCA at the 3' terminus after transcription.

RNA synthesis and tritium release assay

The plasmid DNAs containing the human serine tRNA (h tRNA^{Ser}) gene (gift of H. Gross, Würzburg, Germany) and the *S. cerevisiae* pre-tRNA^{lle} (y pre-tRNA^{lle}) (gift of H. Grosjean, Orsay, France, and F. Fasiolo, Strasbourg, France) as well as DNAs for m pre-tRNA^{lle}_{UAU}, h tRNA^{lle}_{AAU}, and m tRNA^{Met} were digested with BstN1 and transcribed (25 µL reactions) with T7 RNA polymerase (Promega Corp.) as described, in the presence of 25 µCi [5-³H]-UTP (20 Ci/mmol; Moravsek Biochemicals and Radiochemicals) with 100 µM cold UTP and 1.0 mM, ATP, GTP, and CTP (Chen and Patton 1999).

Before incubation with wild-type or mutant hPus1p or a Lac control protein expressed in bacteria and isolated in the same way as the hPus1p proteins (Chen and Patton 1999), the RNA substrates were heated to 78°C for 2 min and allowed to cool slowly to 37°C (30 to 40 min). Substrate reactions with the Ψ synthases were carried out in 100 mM ammonium chloride, 10 mM DTT, 50 mM Tris (pH 7.5), and 2 mM MgCl₂ (typically, 100 µL reactions for each assay). The concentration of Ψ synthase used was ~40–50 nM. The lengths of incubation are listed in the text or in the tables containing the data. The tritium release assay method is described in detail elsewhere (Cortese et al. 1974; Patton 1991).

Short-term kinetic analysis of wild-type and R116K hPus1p was carried out as described (Arлуison et al. 1999b) at ³H-UTP-labeled substrate (y pre-tRNA^{lle}) concentrations of between 10 and 50 nM and at an enzyme concentration of ~700 nM. Aliquots (10 µL from a 25 µL total) were removed after 1- and 2-min incubation at 37°C and immediately mixed with charcoal to stop the reaction and allow for determination of the ³H released. The specific activity of the ³H-labeled y pre-tRNA^{lle} was changed for these experiments, in that the concentration of the cold UTP was 250 µM, and everything else was as listed above for the other ³H-labeled substrates. The results reported are from two independent assays. The binding studies were carried out as described (Arлуison et al. 1998) using protein concentrations from 20 to 800 nM in 100 µL reactions and the ³H-labeled y pre-tRNA^{lle} substrate at 500 pM.

CMCT Pseudouridine mapping assay

The method for the mapping of Ψ residues has been described previously (Bakin and Ofengand 1998; Baumstark and Ahlquist 2001). Three-hour incubations were performed using the ³H-labeled y pre-tRNA^{lle} substrate with wild-type hPus1p, the R116C and R267K mutants, and a Lac control as described above. A small aliquot was removed to measure the overall progress of the reaction, using the ³H-release assay. The RNA in each reaction was isolated and was treated with either 0, 0.042 M, or 0.167 M CMCT (1-cyclohexyl-3-[2-morpholinoethyl] carbodiimide metho-p-toluene-sulfonate, Aldrich). After treatment with sodium carbonate (4 h, 37°C) to remove all the adducts except those attached to Ψ residues, the RNA samples were used as templates for reverse transcription

using AMV reverse transcriptase (Promega) and a ^{32}P -end-labeled primer (5'-CACAGAACTTCGGAACCG). The *fmole* Sequencing kit (Promega) was used to generate the sequencing lanes for the gels, using γ pre-tRNA^{lle} plasmid DNA as the template.

Thin layer chromatography assay of Ψ formation

To determine the levels of Ψ at positions 27, 30, 34, and 36 in γ pre-tRNA^{lle} the substrate was synthesized as above but α - ^{32}P -NTPs (50 μCi , 3000 Ci/mmol, 25 μL reactions; MP Biomedicals) were used in place of the ^3H -UTP. To determine levels of Ψ at these positions, the substrate was labeled with α - ^{32}P -CTP (for position 27), α - ^{32}P -GTP (for position 30), or α - ^{32}P -ATP (for positions 34 and 36). The concentration of the nucleotides was 1.0 mM, except for the labeled nucleotide, which was 100 μM . The modification reactions were set up as above and the RNA isolated after a 3-h incubation. This isolated RNA was electrophoresed on a denaturing 10% polyacrylamide gel (19:1; 8.3 M urea) and the full-length RNA was eluted from the gel (Sambrook and Russell 2001). The gel-purified RNA was digested with RNaseT₂, prepared as described (Hiramuru et al. 1966; Lichtler et al. 1992), and chromatographed on thin layer plates (cellulose, 10 cm $\times$ 10 cm) using isobutyric acid:ammonium hydroxide:water (66:1:33;v:v:v) in the first dimension and isopropanol:concentrated HCl:water (70:15:15;v:v:v) in the second (Grosjean et al. 2007). The dried TLC plates were exposed to X-ray film to generate an image and the levels of ^{32}P in the spots quantified with a Personal FX PhosphoImager (Bio-Rad).

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