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Cloning and characterization of a mammalian pseudouridine synthase

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ABSTRACT

This report describes the cloning and characterization of a pseudouridine (Ψ) synthase from mouse that we have named mouse pseudouridine synthase 1 (mpus1p). The cDNA is ~1.5 kb and when used as a probe on a Northern blot of mouse RNA from tissues and cultured cells, several bands were detected. The open reading frame is 393 amino acids and has 35% identity over its length with yeast Ψ synthase 1 (pus1p). The recombinant protein was expressed in *Escherichia coli* and the purified protein converted specific uridines to Ψ in a number of tRNA substrates. The positions modified in stoichiometric amounts in vitro were 27/28 in the anticodon stem and also positions 34 and 36 in the anticodon of an intron containing tRNA. A human cDNA was also cloned and the smaller open reading frame (348 amino acids) was 92% identical over its length with mpus1p but is shorter by 45 amino acids at the amino terminus. The expressed recombinant human protein has no activity on any of the tRNA substrates, most probably the result of the truncated open reading frame.

Keywords: mammalian; pseudouridine; RNA modification; tRNA

INTRODUCTION

It is becoming increasingly clear that modifications of RNA play important functional roles (for review, see Auffinger & Westhof, 1998; Grosjean et al., 1998) and a recent compilation listed 93 different modified nucleosides that occur in tRNA, rRNA, mRNA, small nuclear RNA (snRNA), and other small RNAs (Limbach et al., 1994). The most abundant of the modified nucleosides, and once considered the "fifth nucleotide" (Davis & Allen, 1957) is pseudouridine (Ψ). Pseudouridine is important in the function of tRNA (Bjork, 1995) and it has been postulated that Ψ in rRNA takes part in the peptidyl transfer reaction (Lane et al., 1992; Brimacombe et al., 1993). In addition, there is an association of the presence of Ψ with those RNAs that participate in group transfer reactions (Lane et al., 1995). Finally, a recent report has linked a deletion in the Nap57 gene (a ribosomal RNA pseudouridine synthase that is highly conserved from yeast to human) with an X-linked disorder dyskeratosis congenita (Heiss et al., 1998).

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Abbreviations: Ψ: pseudouridine; mpus1p: mouse pseudouridine synthase 1; hpus1p: human pseudouridine synthase 1; pus1p: yeast pseudouridine synthase 1; DTT: dithiothreitol; TLC: thin-layer chromatography; IPTG: isopropyl-thiogalactopyrannoside; NTPs: nucleotide tri-phosphates.

Pseudouridine is converted from uridine enzymatically, after the nucleotide has been incorporated into the RNA (Johnson & Soll, 1970; Bjork, 1995). In bacterial cells and yeast, there are at least three enzymes that modify tRNAs, based on the specificity of the cloned Ψ synthases and the known sites of Ψ formation (Kammen et al., 1988; Nurse et al., 1995; Simos et al., 1996; Becker et al., 1997; Lecointe et al., 1998). However, enzymes that form Ψ have not been cloned and characterized from higher organisms. Pseudouridine synthases show maximum activity when incubated in the presence of a thiol reductant such as dithiothreitol (DTT) or β-mercaptoethanol and a monovalent cation such as NH_4^+ (Kammen et al., 1988). The yeast pseudouridine synthase, pus1p, contains one atom of zinc that is required to retain its native conformation and for binding to tRNA substrates and removal of the zinc causes the enzyme to lose all activity (Arluison et al., 1998). The reaction does not require ATP or Mg^{++}, nor has an RNA component or cofactor been shown to be necessary for the modification of tRNA (Kammen et al., 1988). It has been demonstrated that a class of small nucleolar RNAs act as guide RNAs in the formation of Ψ in ribosomal RNA in yeast and vertebrates (Ganot et al., 1997; Ni et al., 1997).

The families of Ψ synthases include the truA family (PSUI from *Escherichia coli*, pus1p and pus3p from yeast; Kammen et al., 1988; Simos et al., 1996;

Lecointe et al., 1998), the truB family (truB from *E. coli* and pus4p; Nurse et al., 1995; Becker et al., 1997), the rsuA family (rsuA from *E. coli*; Wrzesinski et al., 1995a), and rlu (rluA and rluC from *E. coli*; Wrzesinski et al., 1995b; Conrad et al., 1998). Within the families there is significant homology but there is very little homology between members of the different families other than a putative uridine-binding motif (Koonin, 1996). This is surprising given that all Ψ synthases are required to perform the same function, breaking the C-N glycosidic bond (position 1 of the base), rotating the uridine ring, and forming a C-C glycosidic bond at position 5 (Wrzesinski et al., 1995a; Garcia & Goodenough-Lashua, 1998). A putative rRNA Ψ synthase in the truB family, Cbf5p, which is associated with box H+ACA small nucleolar RNAs, is thought to be the enzyme responsible for Ψ formation on rRNA guided by snoRNAs (Lafontaine et al., 1998). It is highly homologous to the rat protein Nap57 whose homolog in humans is linked to the genetic disorder dyskeratosis congenita (Heiss et al., 1998).

The experiments described in this report are centered around mammalian Ψ synthases that have been cloned from mouse and human, the first from metazoans. The amino acid sequences of the mammalian proteins are highly conserved when compared with yeast pus1p and there is evidence for alternative splicing of the messenger RNA. The expressed recombinant mouse protein, which we have designated mouse pseudouridine synthase 1 (mpus1p), forms Ψ in the anticodon stem of several tRNAs and in the anticodon loop and stem of a tRNA with an intron. The recombinant human protein lacks amino acids at the amino terminus when compared with the mouse protein and has no activity in vitro.

RESULTS

Cloning and expression of mouse pus1p

The published amino acid sequence for pus1p (pseudouridine synthase 1) from yeast (Simos et al., 1996) was used to screen a six-frame translation of the EST database and several clones were identified, purchased, and sequenced. When the sequences were compared, one clone was missing a portion of the 3′ end of the gene after the termination codon, and the other clone had a deletion within the gene but the 3′ untranslated region was intact. The deletion removes amino acids 68 to 113 (see Fig. 1), which contains part of a highly conserved region in the other Ψ synthases in the truA family (see Discussion). A complete sequence was compiled from the mouse EST clones and the sequence of the cDNA predicted an open reading frame of 393 amino acids with 35% identity with yeast pus1p and 92% identity with the putative human pus1p described below (see Fig. 1) (Accession #AF116237 for mouse and #AF116238 for human). The obvious assumption is that this is a pseudouridine synthase that acts on tRNA and probably has a specificity that is comparable with yeast pus1p.

The insert from the mouse EST clone (AA207755) was used to probe a Northern blot of total RNA from mouse tissues and total and poly A+ RNA from a mouse cell line (RAW 264.7, a monocyte-macrophage line). The results are shown in Figure 2, with several bands (~1.4, 1.6, and 4.3 kb) found in the lanes where total RNA was used and at least two prominent bands (~1.4 and 1.6 kb) when poly A+ RNA from the mouse cell line was probed. In a second blot this pattern was identical and persisted even after high stringency washes, but in addition the poly A+ lane showed a weak band in the 4.3 kb region (data not shown). The smaller bands (~1.4 and 1.6 kb) seen in the autoradiograph could be the result of the alternative splicing of the primary transcript to produce the splice variant identified earlier. We are endeavoring to determine the identity of these stable mRNAs.

The next step was to construct a recombinant that could be expressed in *E. coli* to test the putative protein encoded by the open reading frame for pseudouridine synthase activity. The expression vector used was pET16b, which, with the help of engineered primers for PCR (see Materials and Methods), can be used to express a protein in *E. coli* with a poly histidine amino terminus. The 393 amino acids of the mouse pus1 sequence had an additional 21 amino acids at the amino terminus, which has a run of 10 histidine residues (actual additional sequence is MGHHHHHHHH HHSS GHIEGR H). This would result in the recombinant protein (414 amino acids) having the predicted molecular weight of 46,614 Da. The expression of the protein is induced by IPTG and the poly His leader allows for a single-step purification on a Nickel column (see Fig. 3). The effect of induction on the amount of protein that is expressed is shown in the first two lanes of Figure 3. The flowthrough after initial binding demonstrates that most of the mpus1p is bound to the column and little elutes with either of the two washes although other proteins from *E. coli* do elute. At 200 mM imidazole the mpus1p is eluted as a single band migrating at approximately 46,000 Da (Fig. 3).

Specificity of mouse pus1p

Three of the substrates that we have used to initially determine the specificity of mpus1p are from yeast because these in vitro transcription clones were easy to obtain and we wanted to be able to compare the specificity of our enzyme with the known specificity of yeast pus1p. In addition we obtained a human pre-tRNA to use as a mammalian tRNA substrate (see Fig. 4).

Preliminary tests of activity were conducted using the yeast $tRNA^{Ile}$ substrate (Fig. 4) labeled with α-^{32}P-ATP incubated with S100 extracts made from induced

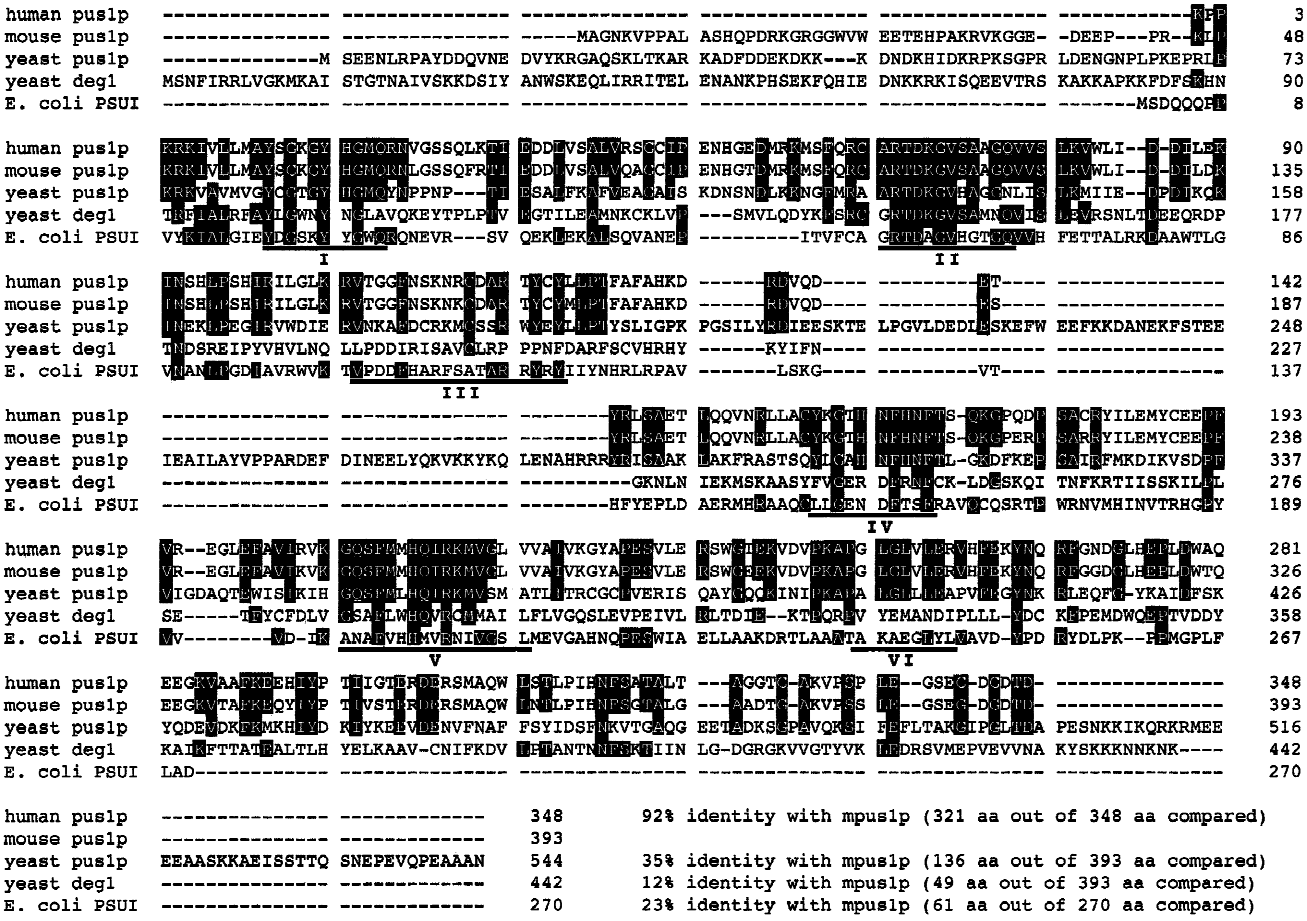

FIGURE 1. The aligned amino acid sequences of human, mouse, and yeast pus1p as well as yeast deg1 and *E. coli* PSUI. The highlighted residues are ones that are conserved between at least three proteins. The dashes (-) in the sequences are gaps to allow for alignment or the absence of sequence.

control *E. coli*, which were transformed with pET16b-β-galactosidase (Fig. 5A), uninduced *E. coli* transformed with pET16b-mpus1 (Fig. 5B), or induced *E. coli* transformed with pET16b-mpus1 (Fig. 5C). The RNA was isolated from the reaction, purified on a denaturing gel, digested with RNase T_2, chromatographed on cellulose plates, and autoradiographed. Note the lack of a spot for Ψ when the S100 was isolated from *E. coli* transformed with the β-galactosidase recombinant (Fig. 5A) and the very faint spot for Ψ when the S100 from the uninduced *E. coli* that had been transformed with the mpus1p expressing recombinant (Fig. 5B). The faint spot can be attributed to a small amount of constitutive expression of mpus1p without induction. A very prominent Ψ spot is apparent when an S100 from the IPTG-induced *E. coli* transformed with the mpus1p expressing recombinant was incubated with the labeled $tRNA^{Ile}$. This tRNA has an intron and the Ψ that is present in Figure 5B,C is indicative of Ψ formed in what will become the anticodon loop, as these are the only Ψs that would be labeled when the $tRNA^{Ile}$ is labeled with α-^{32}P-ATP and is cut with RNase T_2 (see Fig. 4). *E. coli* does not have enzymes that modify $tRNA^{Ile}$ in these positions and therefore the control *E. coli* S100 (Fig. 5A) shows no Ψ. A positive control using HeLa cell (human cervical carcinoma) S100 also formed Ψ on this substrate, as expected (Fig. 5D).

When purified mpus1p (0.3 μg) was used in a reaction (100 μL) with this same labeled $tRNA^{Ile}$, a prominent spot for Ψ was detected on the TLC (Fig. 5e). When this was quantitated using a phosphorimager it was found that 1.5 mol of Ψ was formed per mol of tRNA. The maximum amount of Ψ that could be formed would be 2.0 mol Ψ/mol tRNA.

Next the recombinant mpus1p was tested on several tRNAs labeled with α-^{32}P-UTP. After incubation with the purified mpus1p or β-galactosidase proteins, the RNA was isolated, digested with nuclease P1, chromatographed, and autoradiographed. Nuclease P1 leaves 5′ phosphates and therefore if there was any Ψ formed in the substrate, then a spot would be visible on the resulting autoradiograph shown in Figure 6. Three of the four substrates are recognized by mpus1p, as evidenced by the prominent Ψ spot with

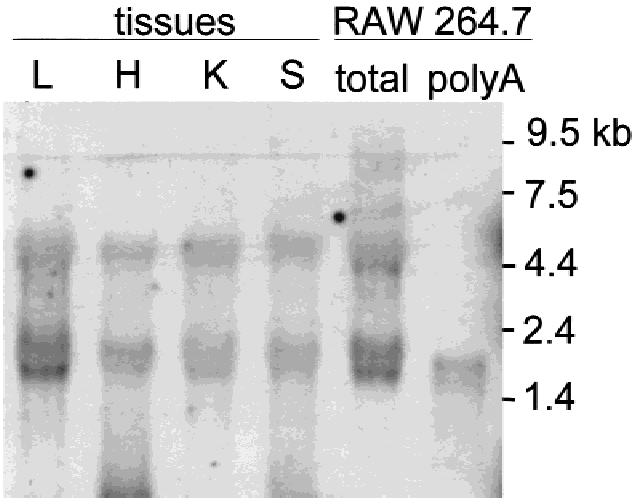

FIGURE 2. Northern blot of mouse RNA probed with mpus1 cDNA. Samples of total (20 μg) or poly A+ RNA (2 μg) were electrophoresed on a 1.2% agarose gel with formaldehyde present (Sambrook et al., 1989). The RNA was blotted to a nylon membrane and hybridized with a ^{32}P-labeled probe random primed from the insert of the mouse EST clone, AA207755. A portion of the autoradiograph is shown with L denoting RNA from liver; H, heart; K, kidney; and S, spleen. The sizes of RNA markers in kilobases are on the right side of the figure.

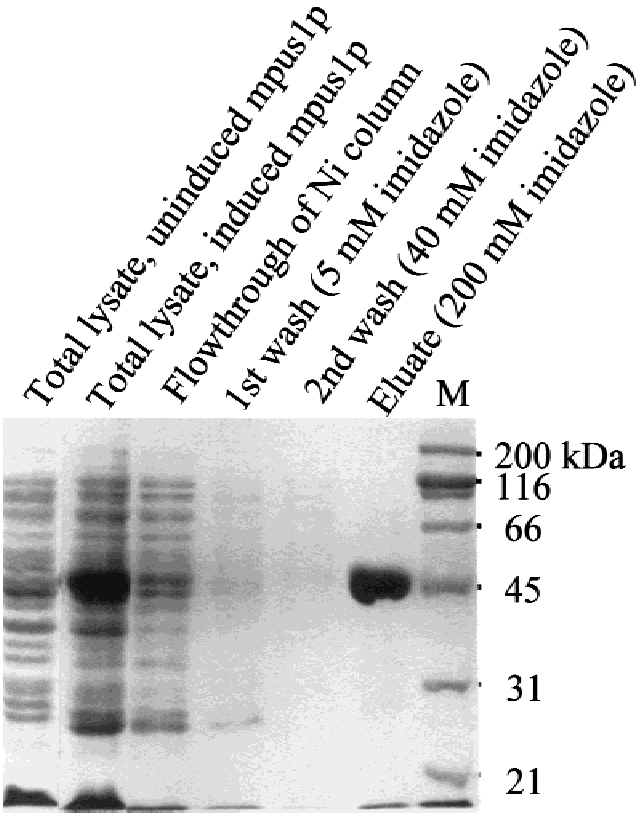

FIGURE 3. Expression and purification of recombinant mpus1p. The method for construction of the expression vector is outlined in Materials and Methods. *E. coli* were transformed with the expression vector pET16b-mpus1p, grown to an optical density of 0.6, and induced with 1mM IPTG for 3 h. Protein samples were electrophoresed on a 12% polyacrylamide/SDS gel, stained with Coomassie Brilliant Blue, and destained. Total lysates from uninduced and induced cells are shown in the first two lanes. The nonbound fraction and washes as well as the eluate from the Ni^{2+} column are also shown. M: markers with the weights in kiloDaltons on the right.

the yeast tRNA^{Ile} and tRNA^{Val} and human pre-tRNA^{Ser,} but yeast tRNA^{Phe} was not recognized. The quantitation from this experiment is given in Table 1 and it can be seen that with the β-galactosidase protein, expressed in *E. coli* and purified in the same way as mpus1p, there is no Ψ formed in any substrate. Therefore no residual pseudouridine synthases from *E. coli* are copurifying with the recombinant proteins chromatographed on the Ni columns.

Yeast tRNA^{Phe} has only two Ψs, one at position 39 and the other at position 55. It would appear that mpus1p does not recognize either of these positions, as no Ψ was formed with this substrate. The other substrates differ from the tRNA^{Phe} by having sites at positions 27/28, and sites in the anticodon loop and the acceptor stem that can be modified (see Fig. 4). With the human pre-tRNA^{Ser} there are three sites of Ψ formation and our results show that approximately one site is modified (0.7 mol Ψ /mol tRNA). The possible sites of modification are at positions 28, 39, and 55. Therefore the most likely site modified on the pre-tRNA^{Ser} is position 28. Based on the results given in Table 1, it would appear three sites are being modified in yeast tRNA^{Ile} and the possibilities are at positions 27, 34, 36, and 67. Position 27 is most likely modified, as the comparable position is modified in pre-tRNA^{Ser} and we have already seen in Figure 5 that the anticodon of tRNA^{Ile} is modified. Therefore it is most likely that mpus1p modifies positions 27, 34, and 36 in vitro. The presence of the intron in tRNA^{Ile} has been shown to be necessary for the formation of Ψ at positions 34 and 36 (Szweykowska-Kulinska et al., 1994), but we have not tested whether that is the case in vitro. Finally, with yeast tRNA^{Val} there are Ψs at positions 27, 32, and 55 (see Fig. 4). With 1.3 mol Ψ/mol tRNA formed, the most likely site is position 27, with the possibility that substoichiometric amounts of Ψ are being formed at position 32 or potentially at position 67, although there is no Ψ at that position normally (see Fig. 4).

The modification of specific positions was verified by labeling the three tRNA substrates that have been shown to be modified by mpus1p with various labeled NTPs and assaying for the presence or absence of Ψ with RNase T_2 and the two-dimensional TLC assay. Yeast tRNA^{Ile} was labeled with α-^{32}P-CTP and three positions could be tested at 27, 55, and 67. The tRNA^{Ile} was first digested by RNase T_1 and the 5 and 16 mers eluted from a denaturing polyacrylamide gel before RNase T_2 digestion. In Table 2 it can be seen that Ψ was only found in the 5 mer that contains position 27 with 0.9 mol Ψ /mol

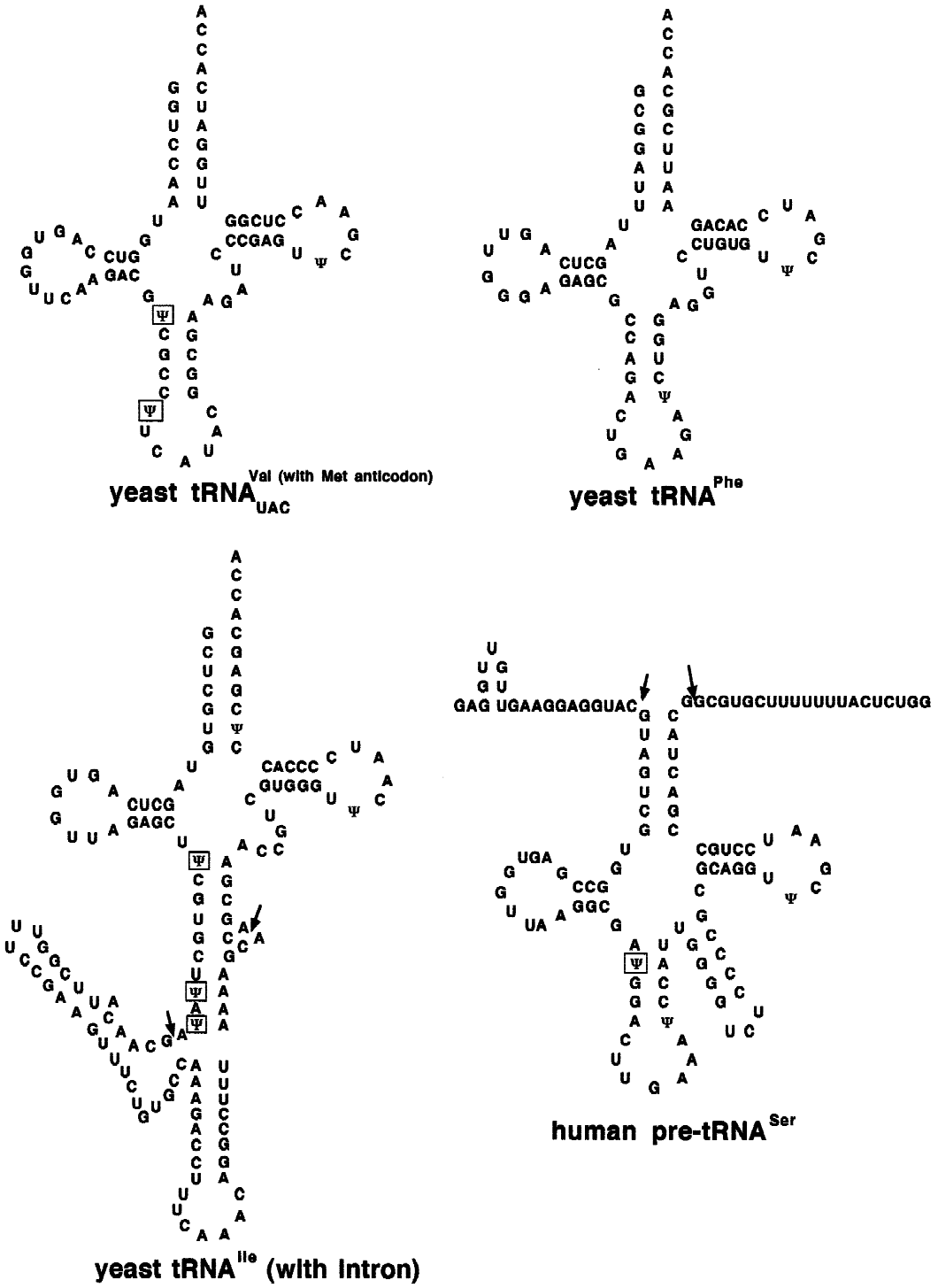

FIGURE 4. Primary and secondary structure of the four tRNA substrates used in the experiments. These are the sequences of the RNA transcripts made in vitro except that the synthesized transcripts contain no Ψ. The Ψs that are boxed are modified by mpus1p in vitro. The other Ψs in the tRNAs are not modified by mpus1p in vitro. The arrows in $tRNA^{Ile}$ molecule indicate the borders of the intron and the arrows in pre-$tRNA^{Ser}$ indicate the 5′ and 3′ end (without CCA) of the mature $tRNA^{Ser}$.

tRNA formed. Therefore there is no Ψ formation at positions 55 or 67 in vitro in this tRNA substrate with mpus1p. As expected when *E. coli* S100 was used, the results are the opposite; there was formation of Ψ in the 16 mer because position 55 would be modified and there was no modification at position 27 (5 mer).

When the yeast $tRNA^{Ile}$ was labeled with α-^{32}P-ATP to test positions 34 and 36, then 1.5 mol Ψ/mol tRNA was

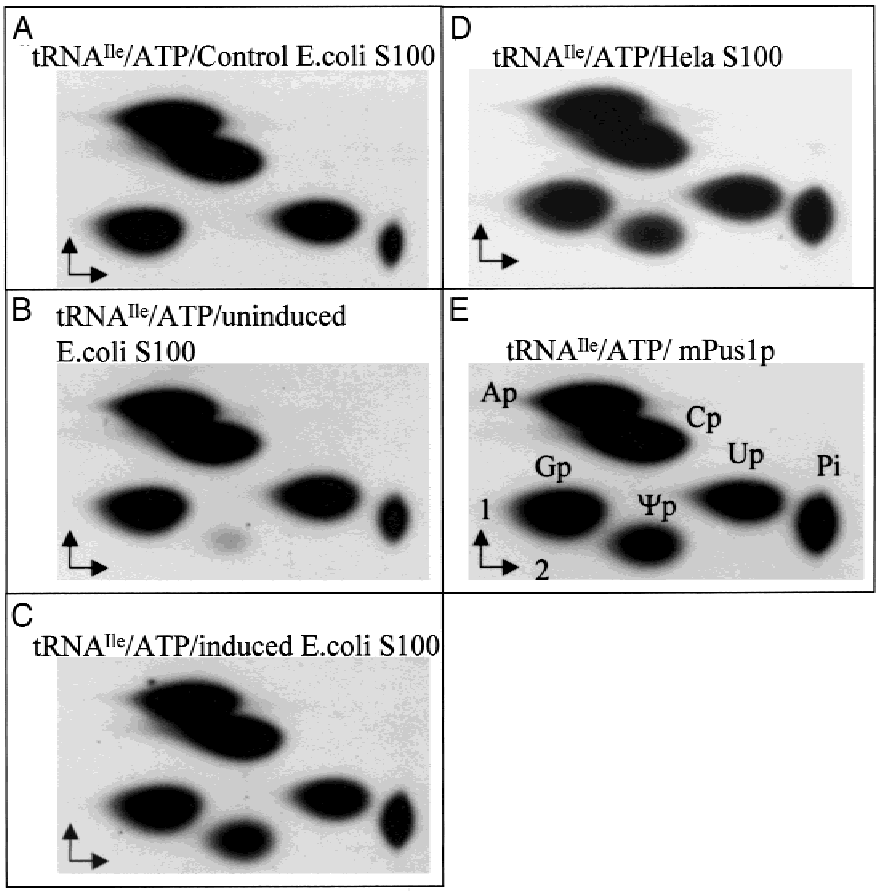

FIGURE 5. Mpus1p has Ψ synthase activity. Autoradiograms of TLC plates obtained after chromatography of RNase T_2 hydrolysates of α-^{32}P-ATP-labeled intron-containing yeast tRNA^{Ile} incubated with different *E. coli* S100, or HeLa S100 extracts or purified mpus1p for 45 min at 37 °C. The identification of the digestion products and the direction of migration (arrows) for each dimension are marked in **E**. **A**: Result obtained after incubation of substrate with a control *E. coli* S100 extract that was prepared from *E. coli* expressing β-galactosidase induced with 1 mM IPTG. **B**: Result obtained after incubation with an *E. coli* S100 extract that was prepared from cells transformed with pET16b-mpus1 but was not induced. **C**: Result obtained after incubation with an *E. coli* S100 extract that was prepared from cells transformed with pET16b-mpus1 and induced with 1 mM IPTG for 3 h. **D**: Result obtained after incubation with HeLa S100 extract. **E**: Result obtained after incubation with purified mpus1p (0.3 μg/100 μL).

formed (these are results from the TLCs shown in Fig. 5). But no Ψ was formed at these positions when an S100 from *E. coli* was incubated with the substrate, because *E. coli* does not have an activity that modifies these positions. The S100 from HeLa cells does modify these positions because human cells should have this activity.

With the yeast tRNA^{Val} labeled with α-^{32}P-CTP, there are only two positions tested, at 27 and 55, and be-

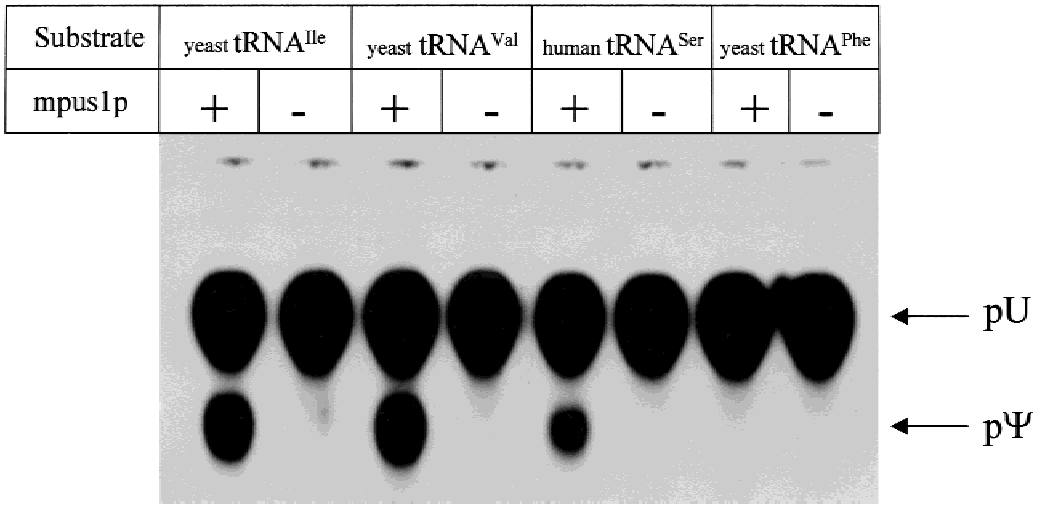

FIGURE 6. Mpus1p activity on several tRNA substrates. Autoradiograms of a TLC plate obtained after chromatography of nuclease P1 hydrolysates of in vitro α-^{32}P-UTP-labeled tRNA substrates incubated (45 min at 37 °C) with either mpus1p (0.3 μg/100 μL) labeled with a + above the lane or a control protein β-galactosidase (0.3 μg/100 μL) labeled with a − above the lane. The positions of pU and pΨ are indicated on the right.

TABLE 1. Results of TLC-^{32}P-labeled substrate assay.[a]

Substrate (anticodon)	Number of Ψ in native molecule (positions)	Recomb. mpus1p (mol Ψ/mol tRNA)	Recomb. β-gal[b] (mol Ψ/mol tRNA)
yeast tRNA^{Phe} (GAA)	2 (39,55)	<0.1	<0.1
yeast tRNA^{Ile} (UAU)+intron	5 (27,34,36,55,67)	2.9	<0.1
yeast tRNA^{Val} (UAC)	3 (27,32,55)	1.3	<0.1
human pre-tRNA^{Ser} (UGA)	3 (28,39,55)	0.7	<0.1

[a]The pU and pΨ spots on a TLC (autoradiograph shown in Fig. 6) were quantified by a phosphorimager using ImageQuan 3.0 Software. The values correspond to Ψ mol formed (±0.1) per mol of tRNA after 45 min incubation at 37 °C with 0.6 μg of protein in each reaction.

[b]The β-galactosidase control is a protein that has a His leader and was isolated from *E. coli* transformed with pET16b-βgal and purified on a nickel column in the same way as the mpus1 protein is purified.

cause position 55 is not modified by mpus1p, we are only testing for position 27. The amount of Ψ formed was 0.8 mol Ψ/mol tRNA. When yeast tRNA^{Val} was labeled with α-^{32}P-UTP and the RNA digested with RNase T_2 to test for Ψ formation at position 32 (or potentially 67), the TLC shows a small amount of Ψ formed. There was no Ψ formed on this substrate when *E. coli* S100 was used because these positions are not modified in *E. coli* tRNA. Therefore it would appear that position 32 (or potentially position 67) is recognized at low efficiency by mpus1p in vitro. However it was shown earlier that Ψ was not formed at position 67 in the yeast tRNA^{Ile} substrate, where there is Ψ formed in vivo.

When human pre-tRNA^{Ser} was labeled with α-^{32}P-GTP, the formation of Ψ at position 28 was tested and 0.8 mol Ψ/mol tRNA were formed when mpus1p was incubated with the substrate. But when this labeled substrate was incubated with S100 from *E. coli,* there was no Ψ detected, as expected. If the pre-tRNA^{Ser} was labeled with α-^{32}P-CTP, Ψ formation at positions 39 and 55 would be checked. There was no formation of Ψ by mpus1p and because there was no Ψ formed in yeast tRNA^{Phe} that has Ψ at these positions (see Fig. 6), none was expected. However, the S100 from HeLa cells did form Ψ (1.6 mol Ψ/mol tRNA), as did the S100 from *E. coli*, as expected.

In summary, mpus1p modifies positions 27/28 in the pre-tRNA^{Ser}, tRNA^{Val}, and tRNA^{Ile} substrates and positions 34, 36 in an intron containing tRNA^{Ile} substrate in vitro at stoichiometric amounts. In addition there are low amounts of Ψ formed at position 32 (or potentially 67) in tRNA^{Val} (UAC). The enzyme accomplishes this without the use of an RNA cofactor and without added ATP.

Cloning of human pus1p

Several human EST clones (I.M.A.G.E. Consortium; Lennon et al., 1996) had very high amino acid identity with yeast pus1p and one was used to screen a HeLa cDNA library (Stratagene). The 5′ end of the longest clone was extended using 5′ RACE (rapid amplification of cDNA ends) and the longest open reading frame obtained predicted a protein of 348 amino acids with high homology (92% identity) to mouse pus1p (see Fig. 1). When this open reading frame identified in the human cDNA was used to construct an expression plas-

TABLE 2. Quantitation of Ψ formation in specific positions of in vitro transcribed tRNAs.

Substrate (anticodon)[a]	[α-^{32}P]NTP	Tested (position)	Control *E. coli* S100	HeLa S100	Recomb. mpus1
yeast tRNA^{Ile} (UAU)+intron	ATP	34,36	−	++	1.5
yeast tRNA^{Ile} (UAU)+intron/5mer[b]	CTP	27	−	nd	0.9
yeast tRNA^{Ile} (UAU)+intron/16mer[b]	CTP	55,67	0.9	nd	−
yeast tRNA^{Val} (UAC)	CTP	27,55	nd	nd	0.8
yeast tRNA^{Val} (UAC)	UTP	32,(67)	−	+	+
human pre-tRNA^{Ser} (UGA)	GTP	28	−	++	0.8
human pre-tRNA^{Ser} (UGA)	CTP	39,55	++	1.6	−

[a]The various labeled tRNA species were incubated with a control *E. coli* S100 extract, HeLa S100 extract, or purified mpus1. The numbers correspond to Ψ mol formed (±0.1) per mol of tRNA after 45 min incubation at 37 °C. The Ψp spots were quantified by PhosphorImager using ImageQuan 3.0 software. nd: the specific experiment was not done; −: no significant radioactivity higher than background was present at the presumed Ψp spot; ++: positive results but not quantified; +: there was significant radioactivity higher than background present at the presumed Ψp spot, but the number of Ψ mol formed per mol of tRNA is very low (<0.1).

[b]tRNA was first digested with RNase T1 and the 5 mer and 16 mer fragments after T1 digestion were purified from a gel and then subjected to RNase T_2 digestion.

mid (starting with the Met residue, amino acid 11 in Fig. 1), the resultant recombinant human pus1p had no activity on any of the substrates that were used with the mpus1p (data not shown). The recombinant human protein was found in inclusion bodies in the *E. coli* and was more difficult to purify because it needed to be denatured and renatured during purification. Because the sequence of the human protein is so conserved relative to the mouse sequence, the additional amino acids at the amino terminus or some portion of this extension are important for at least the correct folding of the enzyme. We are in the process of cloning additional human cDNAs with extensions on the 5′ end using the RACE technique to obtain a longer open reading frame that will allow us to express an active protein.

DISCUSSION

A number of pseudouridine synthases that recognize tRNA have been cloned from yeast since the sequencing of the yeast genome (Simos et al., 1996; Becker et al., 1997; Lecointe et al., 1998). Of these, mouse pus1p has a specificity that is most like yeast pus1p that forms Ψ at positions 27/28 and 34 and 36 in $tRNA^{Ile}$ with an intron (Simos et al., 1996) in vitro. The results presented in this article show that mouse pus1p has the same specificity in vitro. There is also evidence that mpus1p modifies the $tRNA^{Val}$ substrate at position 32 or possibly 67 but at low amounts. Additional sites are modified by yeast pus1p at positions 26, 65, and 67 in vivo but not in vitro (Motorin et al., 1998). Mouse pus1p also does not appear to modify these additional sites in vitro and because the yeast in vivo results depended on a knockout of the PUS1 gene, testing whether mpus1p modifies positions 26, 65, and 67 will await the functional knockout of the PUS1 gene in mice.

We realize that none of our substrates were from mouse, but our results point to the fact that this enzyme is flexible in its recognition of substrates. As long as the tRNA has the correct structure and the uridines to be modified are presented in the correct way, mpus1p can modify the uridine whether it is in a yeast tRNA or a mammalian tRNA. On the other hand when these substrates have other potential sites for the formation of Ψ, mpus1p does not modify those sites. We will be testing additional mammalian substrates and it will be interesting to compare the rates of Ψ synthesis in mammalian versus yeast tRNAs. One interesting experiment might be to replace the yeast PUS1 gene with the mouse PUS1 gene (cDNA) and determine if all the activities of yeast pus1p can be recovered in those cells expressing mouse pus1p instead. Because most of the tRNA substrates tested were from yeast, one would predict that the mouse enzyme will probably substitute for the yeast pus1p in most, if not all, activities.

When the amino acid sequences of other members of the truA family and mammalian pseudouridine synthases are compared, several interesting points can be made. First, there has been a case made that the cysteine residues are important for catalysis (Kammen et al., 1988), based on the fact that thiol specific reagents are required for activity in *E. coli* PSUI. However, mutagenesis evidence where the three cysteine residues in PSUI were changed to either serines or alanines showed that even when all three of the cysteines in the same molecule were changed to alanine, the enzyme is still active, with only a small change in K_m and V_{max} for wild-type versus the triple mutant (Zhao & Horne, 1997). Recently it was suggested that at least one of the cysteines in yeast pus1p might be involved in the complexing of a zinc atom (Arluison et al., 1998). Several cysteines and histidines were identified as possible sites of chelation due to conservation between yeast pus1p, pus2p, and pus3p (Arluison et al., 1998). The cysteine at position 162 (domain III in Fig. 1) and the histidine at position 258 (in domain V in Fig. 1) in the mouse pus1p are conserved in the mammalian enzymes (see Fig. 1) and were identified as possible critical residues for the binding of zinc (Arluison et al., 1998). In the mouse pus1p sequence, the only cysteine that is conserved is the one at residue 162 (Fig. 1).

On the other hand, it was recently shown that an aspartate residue at position 60 of *E. coli* PSUI was absolutely essential for the activity of the enzyme, because when it was changed to alanine, serine, glutamic acid, asparagine, or lysine, there was no detectable activity (Huang et al., 1998). There is an aspartate residue in a highly conserved region RTDKGV (amino acid residues 110–115) that is perfectly conserved between mouse, human, and yeast pus1p and yeast deg1 (see domain II in Fig. 1). The PSUI (*E. coli*) sequence in this region is **RTD**A**GV** and so it will be important to determine if this aspartate is also critical in the mouse enzyme.

The domains identified for members of the truA family (Lecointe et al., 1998) have been included in Figure 1. For all six domains there are conserved amino acids in the mammalian Ψ synthases; however, some are more conserved than others (see above). The sequence identity of mouse pus1p with yeast pus1p and *E. coli* PSUI is quite high, whereas the homology with deg1 is relatively low. It should be pointed out that the homology of mpus1p is the highest with yeast pus1p, an enzyme with which it shares a similar sequence specificity. The homology is not as good with deg1 from yeast and *E. coli* PSUI, which do not share the same substrate sequence specificity. One could consider the conserved sequence between mouse pus1p and yeast pus1p as a core sequence that would be necessary to define the specificity shared by these two synthases.

These examples of homology bring up the point of possible alternative splicing that was identified in the mouse cDNAs sequenced (see Results). In one cDNA a portion of the protein coding sequence is missing

from amino acids 68 to 113 and the reading frame is left intact. This would mean that the aspartate at position 112 would be missing from a protein translated from this message. Given the results discussed earlier concerning the necessity for this aspartate, we anticipate that the protein would have no pseudouridine synthase activity. This deletion occurs at a point where an intron/exon border was identified in the human cDNAs as a consequence of sequencing a clone that retained an intron (data not shown). We are currently determining if this and other examples of alternative splicing are found in other tissues. The genomic DNA will be cloned and sequenced to understand how these alternative splicing events are occurring. In addition we will ascertain whether these alternatively spliced forms are differentially expressed and whether the protein isoforms have differences in activity or specificity when compared with mpus1p.

A number of groups that have cloned genes encoding pseudouridine synthases have noted that there is little homology between members of the different families such as between truA, truB, rsuA, and rluA (Kammen et al., 1988; Nurse et al., 1995; Wrzesinski et al., 1995a, 1995b; Simos et al., 1996; Becker et al., 1997; Lecointe et al., 1998). All these enzymes recognize and bind to RNA and then form Ψ at specific positions, so one would expect stretches of homology that would define components of the catalytic core or binding pockets (Wrzesinski et al., 1995a; Koonin, 1996; Garcia & Goodenough-Lashua, 1998). The representatives of the different families may be examples of convergent evolution and it will be very interesting to compare the structures of these different proteins when they are finally solved.

When the genes for PSUI in *E. coli* and PUS1, PUS3, and PUS4 in yeast were deleted, it was found that they were not essential for viability (Kammen et al., 1988; Simos et al., 1996; Becker et al., 1997; Lecointe et al., 1998), although they grow rather slowly in some cases. It would appear, however, that CBF5, a pseudouridine synthase that acts on rRNA in yeast, is required for growth, although Cbf5p may have functions other than just being a pseudouridine synthase (Lafontaine et al., 1998). The Cpf5p homolog in humans has been linked with the X-linked disorder dyskeratosis congenita (Heiss et al., 1998). It is possible that if the gene for mpus1 was knocked out in the mouse, a phenotype would be seen, as this is a multicellular animal and a loss of efficiency in the translation process, a phenotype seen with hisT (truA) mutants in *E. coli* and *Salmonella* (Negre et al., 1988; Tsui et al., 1991), might have a disastrous effect on development. One of the long term goals for this work is to answer this question with a knockout of PUS1 in mice. Of course we would like to see an effect of the loss of this pseudouridine synthase, but a lack of phenotype with confirmation of the loss of the gene product would also be interesting.

MATERIALS AND METHODS

Materials

Mouse and human I.M.A.G.E. Consortium (LLNL) cDNA clones (mouse: AA207755 and AA017902; human: 40650 and 415887; Lennon et al., 1996) were purchased from Research Genetics, Inc. and Genome Systems and the inserts were sequenced (Sanger et al., 1977). We used the Baylor College of Medicine website (http://kiwi.imgen.bcm.tmc.edu:8088/search-launcher/launcher.html) to accomplish this initial screen. The human embryonic brain cDNA library was purchased from Clonetech and the HeLa cDNA library was purchased from Stratagene. The yeast $tRNA^{Phe}$ (GAA) clone was obtained from Dr. O. Uhlenbeck (Sampson & Uhlenbeck, 1988), yeast $tRNA^{Val}$ (with a Met anticodon UAC) and $tRNA^{Ile}$ (UAU) were obtained from Dr. F. Fasiolo (Szweykowska-Kulinska et al., 1994; Lecointe et al., 1998), and a human pre-$tRNA^{Ser}$ (UGA) was obtained from Dr. S. Altman (pUC19pSer; unpubl.).

Library screening and Northern blot hybridization

The human HeLa cDNA library was screened with random primed insert (labeled with 3,000 Ci/mmol α-^{32}P-dCTP) isolated after restriction digestion of the human EST clone (40650) as described (Sambrook et al., 1989). Inserts of recombinants thus identified were sequenced as described (Sanger et al., 1977). Total mouse RNA was prepared from various tissues or RAW 264.7 cells (mouse monocyte-macrophage line) as described (Chomczynski & Sacchi, 1987) and poly A+ RNA was isolated by oligo dT chromatography (Sambrook et al., 1989). The RNA was electrophoresed on a 1.2% agarose gel with formaldehyde as the denaturant. The RNA was transferred to nylon and was probed with random primed (Sambrook et al., 1989) insert from the mouse EST clone (AA207755).

Expression and purification of recombinant mpus1p

The open reading frame of the mouse protein was amplified by polymerase chain reaction (PCR) using the primers left (AACATATGGCTGGAAACAAGGTGCC) and right (GGATC CTCGAGTCAGTGGCAGGATCTACAGTT) with the mouse EST clone AA207755 as the template. The PCR product was cloned into the pGEMT vector (Promega). The resultant plasmid (pGEMT-mpus1) was sequenced and then digested with *Nde*1 and *Xho*I. The insert was cloned into pET16b (Novagene) that was digested with *Nde*1 and *Xho*1 and the junction regions of the recombinant were sequenced. The recombinant generated (pET16b-mpus1) has an open reading frame that includes a leader of 21 amino acids (MGHH HHHHHH HHSSGHIEGR H) at the amino terminus of the mouse sequence of 393 amino acids shown in Figure 1. *E. coli* strain BL21 (DE3) pLysS was transformed with pET16b-mpus1p, grown at 37 °C to an optical density of 0.6, and induced with 1 mM isopropyl thio-galactopyrannoside (IPTG) for 3 h at 37 °C. Cells were lysed by freeze-thawing once followed by sonication in lysis buffer (50 mM $KHPO_4$ [pH 8.0],

250 mM NaCl, 10 mM β-mercaptoethanol, 0.1% triton-X 100) on ice. The crude cell lysate was centrifuged at 100,000 $\times$ *g* for 1 h in a SW41Ti at 4 °C. The resultant supernatant (the *E. coli* S100 fraction) was directly chromatographed on a Ni^{2+}-NTA column (Qiagen). The protein was bound to the column in 5 mM imidazole in lysis buffer and washes of 5 mM (8 vol) and then 40 mM (8 vol) imidazole in lysis buffer were applied. The bound mpus1p was eluted with 200 mM imidazole in lysis buffer and dialyzed against 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT. The control protein, β-galactosidase, was purified from S100 in the same manner after transformation of *E. coli* with a pET16b-βgal expression plasmid (Novagene) and induction of expression with 1mM IPTG. After dialysis the protein concentrations were determined and glycerol was added to 50%, DTT to 5 mM, and stored at −20 °C.

In vitro transcription and labeling of tRNA substrates

The tRNA substrate plasmid DNAs were digested with *Bst*N1 (yeast Val, Ile, and Phe tRNAs) or *Ava*1 (human Ser tRNA) and transcribed with T7 RNA polymerase as described (Melton et al., 1984). The sequences of all the tRNA transcripts made in vitro are given in Figure 4 except the transcripts were completely unmodified. The label used varied with the needs of the experiment, but typically 50 μCi of α-^{32}P-labeled nucleotide (800 Ci/mmol) was used in the reaction with the labeled nucleotide at 50 μM final concentration (after the addition of cold nucleotide) and the other NTPs were at 250 μM.

Assays for Ψ formation

The conditions for all reactions, whether purified mpus1 or S100 extracts were used, are 100 mM ammonium chloride, 10 mM DTT, 50 mM Tris (pH 7.5), and 2 mM $MgCl_2$. Between 0.3 and 0.6 μg of the recombinant mpus1p were used in reactions and when S100 or β-galactosidase were used, an equal amount of protein was added. Approximately 0.6 pmol (1×10^6 cpm; 14 ng) of the tRNA substrate was used in each reaction. The reaction was digested with Proteinase K (50 μg/mL) in 0.5% SDS at 37 °C for 1 h, extracted with phenol:chloroform (1:1) once, made 0.3 M sodium acetate (pH 5.2), and precipitated with 2.5 vol of 95% ethanol at −20 °C with 10 μg of cold yeast tRNA as carrier. The RNA was purified on a 13% acrylamide (19:1; acryl:bis)/8.3 M urea gel prior to digestion with RNase T_2 or nuclease P1. In some cases the RNA was first digested with RNase T_1, the products electrophoresed on a 20% acryl/8.3 M urea gel, and the RNase T_1 fragments isolated from the gel before RNase T_2 digestion. After nuclease digestion the reaction products were chromatographed on thin layer plates (cellulose, Kodak) using isobutyric acid: 25% NH_4OH:water (50:1.1:28.9; v/v/v) in the first dimension and isopropanol:concentrated HCl:water (70:15:15; v/v/v; Nishimura, 1972) in the second dimension for two-dimensional TLC and just the latter solvent system for one-dimensional TLC (Patton, 1991, 1993, 1994). The TLC plates were autoradiographed using XAR-5 film (Kodak) and the spots quantitated using a phosphorimager and ImageQuan 3.0 Software (Molecular Dynamics).

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