Pseudouridine Synthase 3 from Mouse Modifies the Anticodon Loop of tRNA†

Jianguang Chen and Jeffrey R. Patton*

Department of Pathology, School of Medicine, University of South Carolina, Columbia, South Carolina 29208

Received May 16, 2000; Revised Manuscript Received August 15, 2000

ABSTRACT: A cDNA encoding mouse pseudouridine synthase 3 (mPus3p) has been cloned. The predicted protein has 34% identity with yeast pseudouridine synthase 3 (Pus3), an enzyme known to form pseudouridine at positions 38 and 39 in yeast tRNA. The cDNA is 1.7 kb, and when used as a probe on a Northern blot of total RNA from mouse tissues or cells in culture, a band at 1.8 kb was observed. The open reading frame codes for a protein of 481 amino acids with a predicted molecular mass of 55 552 Da. When mPus3p was in vitro translated and used in reactions with tRNA substrates from both yeast and humans, uridines at position 39 were modified to pseudouridine. In a tRNA substrate with a uridine at position 38 (human $tRNA^{Leu}$), there was very slight formation of pseudouridine at that position after incubation with mPus3p.

Pseudouridine (Ψ)[1] is an abundant modification found in stable RNAs such as tRNA (*1*), rRNA (*2*), and small nuclear RNA [snRNA; (*3*)] and was at one time considered the 'fifth nucleotide' found in RNA (*4*). Because of the additional imino proton on the base, Griffey et al. (*5*) point out that Ψ is potentially more versatile in its hydrogen bonding interactions than uridine. The presence of Ψ has been shown to stabilize base stacking in oligonucleotides (*6*) and to contribute to the stability of hydrogen bonding (*7*−*9*). Pseudouridine appears to be necessary for the alternate reading of codons (*10, 11*) and to prevent mischarging of the tRNA (*12*). The presence of Ψ is necessary for the function of U2 snRNA in the splicing of pre-messenger RNA in *Xenopus* oocytes (*13*).

The formation of Ψ in RNA is a posttranscriptional event (*14*) carried out by pseudouridine synthases. A number of these enzymes have been isolated from prokaryotes and eukaryotes (*15*−*28*). The synthases are organized into families (*29*) with the truA family currently having the most members. Members of this family modify tRNA and include PSUI from *E. coli* [also called truA or hisT (*20*)] which modifies positions 38, 39, and 40. Other members are Pus1p from yeast (*24*) and mPus1p from mouse (*17*) which modify positions 27 and 28 and the anticodon of intron-containing tRNAs. Pus1p has been shown to modify additional positions in the acceptor stem in vivo (*30*) and one position in U2 snRNA in vivo and in vitro (*31*). Finally, Pus3p [also known as Deg1 (*22*)] modifies positions 38 and 39 in yeast tRNAs and is also a member of the truA family.

Many of the pseudouridine synthases that have been cloned have also been tested to see if they are essential genes. The absence of PSUI in *E. coli* was at first thought to have little effect on growth, but subsequently it was found that PSUI was required for normal growth on uracil-deficient media (*32*). When the genes for the rRNA pseudouridine synthases RluA (*33*), RluC (*18*), or RsuA (*34*) were deleted, there was no effect on the growth of *E. coli* in rich or minimal media. However, when a wild-type strain and an *rluA*- strain were grown together, there was a strong selection against the *rluA*- strain (*33*). Raychaudhuri et al. (*23*) found that if the rRNA pseudouridine synthase RluD was disrupted in *E. coli* the generation time for the mutant was twice that of the wild-type bacteria. This enzyme is responsible for Ψ formation at 1915 and 1917 on 23S rRNA, which are highly conserved (*23*). When the *pus3* gene was disrupted in yeast, it was not lethal, but the growth rate of the yeast is considerably reduced, especially at 37 °C (*22*). When *pus1* and *pus4* were deleted, there was no effect on the growth of the yeast (*16, 24*). However, if the deletion of *pus1* was combined with the deletion of *los1* (a gene involved with the tRNA maturation that is not lethal when deleted alone), the combination was lethal (*24*). Finally, when the gene for CBF5 was mutated in yeast such that there was no in vivo pseudouridylation of rRNA, the cells have a slow growth phenotype, and there were substantially reduced levels of 40S and 60S ribosomal subunits in the cytoplasm (*35*).

As a start in understanding the role of Ψ in more complex systems, we have cloned another Ψ synthase from mammals and designated it mouse pseudouridine synthase 3 (mPus3p). The mouse enzyme has homology with yeast Pus3p (*22*) both in primary sequence and in the position that it modifies on tRNA. The predicted open reading frame (ORF) of mPus3p has 34% identity with yeast Pus3p and exhibits long stretches of identity in areas known to be conserved in members of the truA family. Mouse Pus3p forms Ψ at position 39 in

† This work was supported by a grant to J.R.P. from the National Science Foundation (MCB-9603550) and the Research Development Fund of the University of South Carolina School of Medicine.

* Address correspondence to this author. Phone: (803) 733-3399. FAX: (803) 733-1515. Email: patton@med.sc.edu.

[1] Abbreviations: Ψ, pseudouridine; mPus3p, mouse pseudouridine synthase 3; mPus1p, mouse pseudouridine synthase 1; Pus1p, yeast pseudouridine synthase 1; Pus3p, yeast pseudouridine synthase 3; DTT, dithiothreitol; TLC, thin-layer chromatography; NTPs, nucleotide triphosphates; ORF, open reading frame; snRNA, small nuclear RNA; EST, Expressed Sequence Tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase−polymerase chain reaction; SDS, sodium dodecyl sulfate; TBS-Tween, Tris-buffered saline with 0.1% Tween-20.

10.1021/bi001109m CCC: $19.00 © 2000 American Chemical Society
Published on Web 09/14/2000

several tRNAs in vitro, and slight activity was detected at position 38 on human $tRNA^{Leu}$.

EXPERIMENTAL PROCEDURES

Materials. I.M.A.G.E. Consortium (LLNL) cDNA clones [AA288508, AA387627, AA517185; (*36*)] were purchased from Genome Systems Inc., and the inserts were completely sequenced (*37*). We used the Baylor College of Medicine website (http://www.hgsc.bcm.tmc.edu/SearchLauncher/) to identify the clones using the amino acid sequence for yeast Pus3p (*22*) for comparison. The yeast $tRNA^{Phe}$ clone was obtained from Dr. O. Uhlenbeck (*38*), the yeast $tRNA^{Val}$ (with Met anticodon UAC) clone was obtained from Dr. F. Fasiolo (*22, 39*), the human $tRNA^{Lys3}$ clone was the gift of Dr. R. Marquet (*40*), and the human $tRNA^{Ser}$ and $tRNA^{Leu}$ clones were the gifts of Dr. H. Gross (*41, 42*).

Cloning of mPus3 cDNA, Construction of an Expression Plasmid, and in Vitro Translation. None of the EST clones contained a complete open reading frame (ORF) based on a comparison with the yeast Pus3p amino acid sequence. However, sequences at the putative 5′ and 3′ ends of the message were present in the clones, and oligodeoxynucleotides were generated for use in RT-PCR with mouse RAW264.7 poly A+ RNA (*43, 44*). The forward primer was 5′CATATGGCTGAAAACACAGACAG3′, and the reverse primer was 5′GGATCCTCGAGATTGCTCCCTGGATCCTGTT3′. The fragment generated by RT-PCR was inserted into pGEMT-Easy (Promega) and sequenced (*37*). The *Xho*I−*Nde*I fragment from pGEMT-mpus3 was inserted into pET16b (Novagen) digested with *Nde*I and *Xho*I. The ORF included the additional amino acid sequence MGHHHHHHHH HHSSGHIEGRH at the amino terminus of the mPus3p coding sequence, which would increase the size to 502 amino acids (58 091 Da). The pET16b-mPus3 DNA was used directly in the T7 coupled transcription/translation reaction (TNT T7 Coupled Reticulocyte Lysate System, Promega) to produce mPus3p in vitro using the manufacturer's recommendations. The enzyme was partially purified on a Ni^{2+}-NTA column (Qiagen) as described (*17*) except the elution of the bound protein was accomplished with 0.5 M imidazole in lysis buffer. The eluate was dialyzed against 50 mM Tris (pH 7.5), 2 mM $MgCl_2$, 200 mM NaCl, 1 mM DTT, and 0.5 mM PMSF. The dialysate was concentrated on Centricon 3 columns (Amicon); glycerol was added to 50% and stored at −20 °C. The final concentration of recombinant mPus3p in the preparation by Western blot analysis (see below) was estimated to be between 0.8 and 1.6 ng/μL. This preparation of mPus3p is not pure, and stained gels do not show a band that can be discerned from the background of proteins that bind and elute from the Ni^{2+}-NTA column.

Blots. Total mouse RNA was isolated from liver, kidney, and RAW264.7 cells (mouse monocyte-macrophage line) as described (*45*). The RNA samples (20 μg) were electrophoresed on a 1.0% agarose gel with formaldehyde, transferred to nitrocellulose, and probed with random-primed *Xho*I−*Nde*I insert from the pGEMT-mpus3 clone or GAPDH DNA (*46*). For Western blots, aliquots from the coupled transcription/translation reactions (5 μL of a 50 μL reaction), incubated with or without pET16b-mPus3p DNA, were electrophoresed on a 10% polyacrylamide−SDS gel (*47*) and transferred to nitrocellulose (*46*). The Western blot was blocked with 5% nonfat dry milk in 20 mM Tris (pH 7.6), 137 mM NaCl, 0.1% Tween-20 (TBS-Tween) overnight at 4 °C. Then it was incubated with the primary antibody (Tetra-His Antibody, Mouse IgG_1 monoclonal; Qiagen) for 16 h and then the secondary antibody (sheep anti-mouse horseradish peroxidase conjugated Ig, Amersham/Pharmacia Biotech) for 2 h in blocking buffer. TBS-Tween was used in the washing steps after the incubations with the anitbodies. The ECL Western blotting detection reagents (Amersham/Pharmacia Biotech) were used to detect in vitro translated mPus3p using XAR film (Kodak).

In Vitro Transcription and Labeling of tRNA Substrates. The plasmid DNAs containing the tRNA genes were digested with BstN1, except for the $tRNA^{Lys3}$ plasmid which was digested with EcoT22I (*40*), and all were transcribed with T7 RNA polymerase as described (*48*). The label used varied with the needs of the experiment, but typically for labeling with [5-^{3}H]UTP, 50 μCi of labeled nucleotide was used (12.8 Ci/mmol) with 122 μM as the final UTP concentration. For labeling with α-^{32}P-labeled nucleotide, 50 μCi of [^{32}P]UTP was used (800 Ci/mmol) with 50 μM as the final concentration of UTP. For all the reactions the other, nonlabeled NTPs were at 500 μM. The RNAs were purified on 10% polyacrylamide/8.3 M urea gels before use in assays.

Analyses for Ψ Formation. The 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 $MgCl_2$ (*17*). The tRNA substrates, without enzyme, extract, or DTT, were heated to 65 °C for 2 min and allowed to cool slowly to 35 °C (30−40 min). The amount of in vitro translated mPus3p was one-tenth of a standard coupled transcription/translation reaction (5 μL), estimated from the Western blot to be between 15 and 30 ng of protein for the initial screen of substrates. The standards on the Western blot were known quantities of purified recombinant mPus1p expressed in *E. coli* which also has the same His leader as recombinant mPus3p. Due to dilution during purification, it was estimated that only about 4−8 ng of the partially purified mPus3p was used per reaction. The times of incubation are listed in the text or in the tables containing the data. In some experiments, the ^{3}H-release method was used to analyze for Ψ formation. This method is described in detail elsewhere (*49*), but briefly it involves mixing the incubated sample with Norit A charcoal which binds all counts except those that have been split out to water in the process of forming Ψ (*50*). The released counts are separated from the charcoal and counted. Triplicates were done on each sample by splitting the original sample into three equal parts.

To determine the position of Ψ formation, the ^{32}P-labeled tRNAs were first incubated with partially purified mPus3p, isolated from the reaction, digested with RNase T1, and electrophoresed on a 20% polyacrylamide/8.3 M urea gel. The bands corresponding to particular RNase T1 fragments were identified by autoradiography and eluted from the gel. The purified fragments were digested with RNase T2 and 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 2-propanol/concentrated HCl/water (70:15:15; v/v/v) in the second dimension (*51*). The spots were quantitated using a FX phosphoimager with Quantity One software (BioRad).

```
mouse Pus1p --------------M AGNKVPPALASHQ-- ---------------- -PDRKGRGGWVWEET EHPAKRVKGGEDEEP ----------PRKLP     48
yeast Pus1p --------------M SEENLRPAYDDQVNE DVYKRGAQSKLTKAR KADFDDEKDKKKDND KHIDKRPKSGPRLDE NGNPL---PKEPRLP     73
mouse Pus3p --------------M AENTDRNQIEKLLN- ----------RVKEL EQEVERLKKKKEQAN NIKDSSIRENSLGSG KAKRA---FDFSAHG     62
yeast Pus3p MSNFIRRLVGKMKAI STGTNAIVSKKDSIY ANWSKEQLIRRITEL ENANKPHSEKFQHIE DNKKRKISQEEVTRS KAKKAPKKFDFSKHN     90
E.coli PSUI ---------------- ---------------- ---------------- ---------------- ----------MSDQ ----------QQPP      8

mouse Pus1p KRKIVLLMAYSGKGY HGMQRNLGSSQFRTI EDDLVSALVQAGCIP ENHGTDMRKMSFQRC ARTDKGVSAAGQVVS LKVWLID-DILDKIN    137
yeast Pus1p KRKVAVMVGYCGTGY HGMQVNPPNP---TI ESALFKAFVEAGAIS KDNSNDLKKNGFMRA ARTDKGVHAGGNLIS LKMIIEDPDIKQKIN    160
mouse Pus3p RRHVALKIAYDGWGY QGFASQENTSN--TI EEKLFEALTKTRLVE S-----RQTSNYHRC GRTDKGVSAHGQVIS LDLRSQFPTSRDSED    145
yeast Pus3p TREIALRFAYLGWNY NGLAVQKEYTPLPTV EGTLLEAMNKCKLVP S---MVLQDYKFSRC GRTDKGVSAMNQVIS LEVRSNLTDEEQRDP    177
E.coli PSUI VYKIALGIEYDGSKY YGWQRQNEVR---SV QEKLEKALSQVANEP IT---------VFCA GRTDAGVHGTGQVVH FETTALRKDAAWTLG     86
                             I                                                                  II

mouse Pus1p SHLPSHIRILGLKRV TGGFNSKNKCDARTY CYMLPTFAFAHKD-- ----RDVQDE----- ---------------- ----------     186
yeast Pus1p EKLPEGIRVWDIERV NKAFDCRKMCSSRWY EYLLPTYSLIGPKPG SILYRDIEESKTELP GVLDEDLESKEFWEE FKKDANEKFSTEEIE    250
mouse Pus3p SNLKHEADDLAKEIR YTHILNRVLPADIRV LAWAPVEPSFSAR-- ---FSCLERTYR--- ---------------- ----------     197
yeast Pus3p TNP--------SREIP YVHVLNQLLPDDIRI SAVCLRPPPNFDAR- ---FSCVHRHYK--- ---------------- ----------     223
E.coli PSUI VVANLPG---DIAVR WVKTVPDDFHARFSA TARRYRYIIYNHR-- ---LRPAVLSKG--- ---------------- ----------     135
                                          III

mouse Pus1p ---------------- ---------------- -----SYRLSAETLQ QVNRLLACYKGIHNF HNFTSQKGPREPSAR RYILEMYCEEPFVR-    240
yeast Pus1p AILAYVPPARDEFDI NEELYQKVKKYKQLE NAHRRRYRISAAKLA KFRASTSQYLGAHNF HNFTLGKDFKEPSAI RFMKDIKVSDPFVIG    340
mouse Pus3p ---------------- ---------------- ----YFFPRADLDIA TMNYAADKYVGIHDF RNLCKMDVANGVINF QRTILCAQVQLVAQS    253
yeast Pus3p ---------------- ---------------- ----YIFNGKNLNIE KMSKAASYFVGERDF RNFCKLDGSKQITNF KRTIISSKILPLSET    279
E.coli PSUI ---------------- ---------------- ----VTHFYEPLDAE RMHRAAQCLLGENDF TSER--AVQCQSRTP WRNVMHINVTRHGP-    188
                                                                                IV

mouse Pus1p -------EGLEFAVI KVKSQSFMMHQIRKM VGLVVAIVKGYAPES VLERSWGEEKVDVPK APGLGLVLERVHFEK YNQRFGGDGLHEPLD    323
yeast Pus1p D------AQTEWISI KIHSQSFMLHQIRKM VSMATLITRCGCEVE RISQAYGQQKINIPK APALGLLIEAPVFEG YNKRLEQFG-YKAID    423
mouse Pus3p PGEERRQEPFQLCQF EVISQAFLYHQVRDM MAILFLIGQGMEKPE IIQELLNIQKNPQKP QYSMAVEFPLVLYDC KFENTKWIYDHEVQE    343
yeast Pus3p -----------FYCF DLVGSAFLWHQVRDM MAILFLVGQSLEVPE IVLRLTDIEKTPQRP VYEMANDIELLLYDC KFPEMDWQE-PTVDD    357
E.coli PSUI -----------YVVV DIKANAFVHHMVRNI VGSLMEVGAHNQPES WIAELLAAKDRTLAA ATAKAEGLMLVAVD- -------YP--DRYD    257
                                      V                                                  VI

mouse Pus1p WTQEEGKVTAFKEQY IYPTIVSTER----- --------DERSMAQ WLNTLPIHNFSGTAL G-------------- ----------    371
yeast Pus1p FSKYQDEVDKFKMKH IYDKIYKEEV----- --------DENVFNA FFSYIDSFNKVTGAQ GE-ETADKSGPAVQK SIFEFLTAKGIPGLT    499
mouse Pus3p FNVTHLQQLWANHAV KTHMLYSMLQGLDSV MVTCAAGTKMDEATE WRNIQPPVIKHTSAF VEGVKMRTYKPLMDR PKCQGLESRIRHFVS    433
yeast Pus3p YKAIKFTTATEALTL HYELKAAVCN----- -----IFKDVLPTAN TNNFSKTIINLGDGR GKVVGTYVKLE---- ----------    418
E.coli PSUI LPKPPMGPLFLAD-- ---------------- ---------------- ---------------- ---------- ----------    270

mouse Pus1p ---------------- ----AADTGAKVPSS LEGSEGDGDTD---- ---    393
yeast Pus1p DAPESNKKIKQRKRM EEEEAASKKAEISST TQSNEPEVQPEAAAN ---    544
mouse Pus3p RGRIEHPHLLHKEEI KARRDCADKEENTVV ENPSKRVCIIDAEIN SIA    481
yeast Pus3p ---------------- ----DRSVMEPVEVV NAKYSKKKNNKNK-- ---    442
E.coli PSUI ---------------- ---------------- ---------------- ---    270
```

FIGURE 1: Aligned amino acid sequences of mPus1p, mPus3p, yeast Pus1p, yeast Pus3p, and *E. coli* PSUI. The highlighted residues are conserved between at least three of the five proteins. The dashes (−) in the sequences are gaps to allow for optimal alignment or denote the absence of sequence at the termini. Six regions of homology are indicated by underlining and a Roman numeral.

RESULTS

Databases containing mouse Expressed Sequence Tag (EST) clones were searched for potential mouse sequences that were homologous to the amino acid sequence of yeast Pus3p (*22*). Several EST clones were identified and sequenced, and it was found that none contained a complete ORF when compared with yeast Pus3p. The sequences obtained from the EST clones were used to generate oligonucleotide primers for the synthesis of a Mpus3 cDNA from mouse poly A+ RNA by RT-PCR. The resultant clone (1.7 kb; accession number AF266505) was sequenced, and an ORF of 481 amino acids (55 552 Da predicted) was identified. When this sequence was compared with yeast Pus3p, there was 34% identity (see Figure 1). Within the six regions of homology indicated in Figure 1, the percent homology is considerably higher. For example, in region II there are 11 identical residues out of 13 and the 2 residues that are not identical are similar.

When a Northern blot of total RNA from mouse tissues and a mouse macrophage cell line (RAW264.7) was probed with labeled mPus3 cDNA, a diffuse band at ~1.8 kb (Figure 2) was identified. The blot was reprobed with labeled cDNA for GAPDH as a control for RNA loading. The amount of RNA loaded on the gel was higher for RAW cells when compared with one of the liver samples and the kidney sample (lanes 3 and 5 in Figure 2). The relative levels of GAPDH mRNA quantitated by phosphoimager (see Experimental Procedures) are given at the bottom of the panel for comparison. Even given the unequal loading, the levels of message for mPus3p are significantly higher in RAW cells than in either of the tissues (compare lanes 1 and 2 with lanes 3−5 in Figure 2). However, the mRNA for mPus3p is not very abundant in any source. The exposure of the mPus3 portion of Figure 2 was 8 days with an intensifying screen,

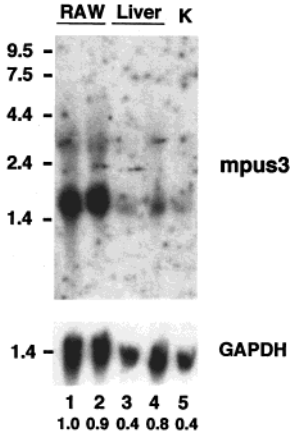

FIGURE 2: Northern blot of total RNA from mouse tissues and cells in culture. Total RNA (20 μg) was electrophoresed on a 1.0% agarose gel with formaldehyde, transferred to nitrocellulose, and hybridized with a mPus3 cDNA probe (top panel) initially and then a GAPDH cDNA probe (bottom panel). The autoradiographs are shown with the sizes of RNA markers in kb on the left. Lanes 1 and 2 contain RNA from mouse RAW264.7 cells; lanes 3 and 4 are separately isolated samples of mouse liver total RNA; lane 5 is total RNA from mouse kidney. The relative levels of GAPDH mRNA, measured by phosphoimager in a separate exposure, are shown at the bottom of the lower panel for comparison.

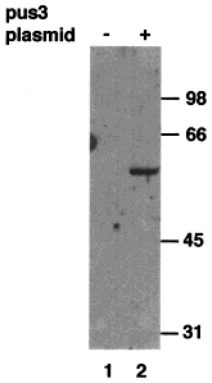

FIGURE 3: Western blot of coupled in vitro transcription/translation reactions. Aliquots of the in vitro transcription/translation reactions without (−, lane 1) and with (+, lane 2) pET16b-mPus3 DNA were electrophoresed on a 10% SDS−polyacrylamide gel (*47*), transferred to nitrocellulose, and reacted with an anti-His monoclonal antibody (primary) and a horseradish peroxidase conjugated sheep anti-mouse antibody. The chemiluminesence was detected on XAR film (see Experimental Procedures), and the positions of molecular weight markers are shown on the right.

whereas the exposure for GAPDH was 1 day without an intensifying screen. In addition, the probe for mPus3 had twice the specific activity of the probe for GAPDH.

To study the specificity of mPus3p, the ORF was cloned into the pET16b vector for expression in *E. coli* as an amino-terminal His-tagged protein. This method was used quite successfully for the expression of mPus1p (*17*); however, no expression of mPus3p was detected in *E. coli*. The recombinant protein was absent from both the supernatant and the pellet after lysis of the cells. Different hosts were used, and conditions of growth were modified, all without positive result. Instead, mPus3p was translated in vitro in a coupled in vitro transcription/translation system. A Western blot of an aliquot of the reactions, with and without the plasmid DNA included, is shown in Figure 3. As expected, there was no reaction with an anti-His antibody in the lane where no DNA was included in the reaction. However, there was a prominent band (estimated by linear regression to be 57 000 Da) in the lane where pET16b-mpus3 DNA was included. The predicted size of the in vitro translated product is 58 091 Da, allowing for the mass of the additional 21 amino acids that compose the His tag in this vector (see Experimental Procedures for the sequence).

To carry out an initial screen of the mPus3p activity, the in vitro translation reactions were used without further purification. As a control for specificity, mPus1p [pET16b-mPus1p, (*17*)] was also translated in vitro in a separate reaction. Aliquots of the reactions were incubated with the substrates that are shown in Figure 4. The substrates were transcribed in vitro in the presence of [5-^{3}H]UTP, and the tRNAs have no modifications prior to incubation with the enzymes. Table 1 shows the results from this experiment. In the column that includes the reticulocyte lysate without any added plasmid DNA, there is a baseline of activity that varies with the substrate used. This is due to a small amount of Ψ synthase activity inherent in the reticulocyte extract. To keep the contribution of the background activities to a minimum, the reactions were incubated only 30 min. Unfortunately, this short incubation, coupled with the fact that there may have been some degradation of the substrates during the reaction, resulted in less than stoichiometric amounts of Ψ being made.

When in vitro translated mPus3p was used in the reactions, there was activity with all the substrates except yeast tRNA^{Val}, which exhibited an activity lower than the level seen with lysate alone. If mPus3p modifes tRNA at positions 38 or 39, this is what would be expected since all of the tRNA substrates used have a uridine at position 39 except tRNA^{Val} (see Figure 4). The observed activity on human tRNA^{Leu} was not significantly higher than the other substrates, even though this substrate has a uridine at both position 38 and position 39 (*41*).

When mPus1p was used with four of the substrates, two were modified, yeast tRNA^{Val} and human tRNA^{Ser}, and two were not, yeast tRNA^{Phe} and human tRNA^{Leu}. Since mPus1p would only be expected to modify positions 27 or 28 in these particular tRNAs in vitro, the results are entirely expected and are a good complement to the results with mPus3p. It has already been shown that mPus1p has no activity with yeast tRNA^{Phe} (*17*) and there is no uridine at position 27 or 28 in human tRNA^{Leu} [Figure 4; (*41*)]. Yeast tRNA^{Val} and human tRNA^{Ser} have been shown to be substrates for mPus1p (*17*).

To reduce the background of activity found in the reticulocyte lysate translated mPus3p, the in vitro translation reaction was subjected to Ni^{2+}-NTA chromatography, and the bound mPus3p was washed, eluted, and concentrated (see Experimental Procedures). This partially purified enzyme preparation and purified mPus1p expressed in *E. coli* (*17*) were used in ^{3}H-release assays with human tRNA^{Leu} and yeast tRNA^{Val}. After a 2 h incubation, mPus3p gave an activity of 0.32 mol of Ψ/mol of tRNA when tRNA^{Leu} was the substrate (Table 2). However, a very low level of activity (0.03 mol of Ψ/mol of tRNA) was observed with yeast tRNA^{Val}, which was expected (see Table 2). The purified recombinant mPus1p gave an activity of 0.40 mol of Ψ/mol of tRNA with yeast tRNA^{Val} and no activity (<0.01 mol of Ψ/mol of tRNA; Table 2) with human tRNA^{Leu}, which is what was expected given the known substrate specificity of mPus1p (*17*). These results confirm that the level of contaminating Ψ synthase activity from the reticulocyte lysate is negligible in the partially purified mPus3p preparation. The observed activity is lower in this experiment and is probably due to the fact that the enzymes are more dilute.

To determine which uridine was modified by mPus3p, two substrates were transcribed in vitro with [^{32}P]NTPs. Human tRNA^{Leu} was labeled with [^{32}P]CTP and UTP in separate reactions, and human tRNA^{Ser} was labeled with [^{32}P]CTP. The tRNAs were incubated with partially purified mPus3p for 1 h, and the RNA was isolated from the reactions. tRNAs labeled with [^{32}P]CTP, that were sham-incubated, were also isolated as controls. The tRNAs incubated with mPus3p were digested with RNase T1 and electrophoresed on a 20% acrylamide/8.3 M urea gel, and RNase T1 fragments were eluted from the gel. The fragments were digested with RNase T2 and chromatographed on TLC plates in two dimensions [see Experimental Procedures; (*51*)]. If mPus3p modified the uridine at position 39 to a Ψ, the modified base would be detected in the 10mer from tRNA^{Ser} (CTP labeled) and the 5mer from tRNA^{Leu} (CTP labeled). The autoradiographs that resulted from these two fragments are shown in Figure 5 and it can be seen that Ψ is found in both fragments. The

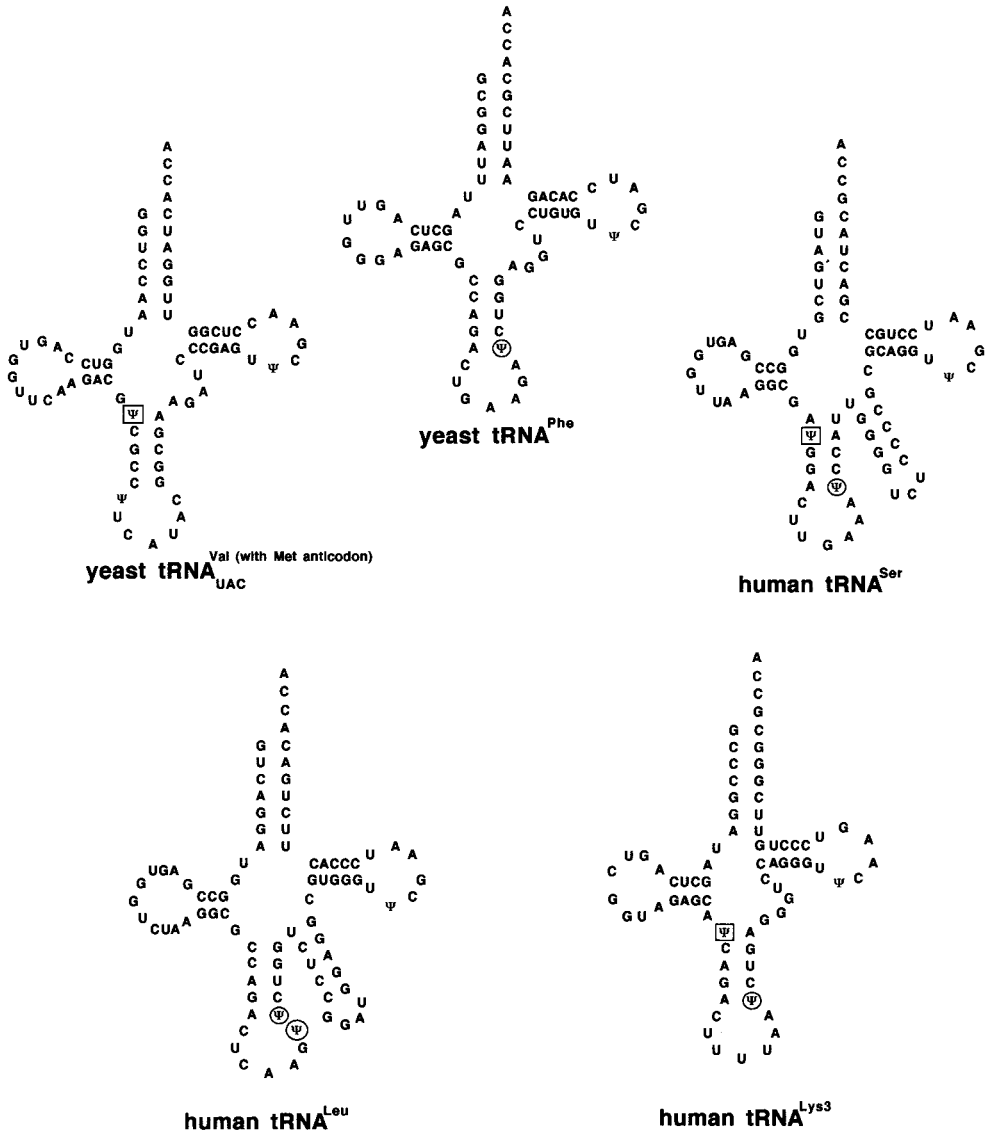

FIGURE 4: Primary and secondary structures of the five tRNA substrates used in the experiments. These are the sequences of the tRNAs that were transcribed in vitro except that there are no modifications on the in vitro transcripts. The Ψs that are boxed would be expected to be modified by mPus1p in vitro, and the Ψs that are circled would be expected to be modified by mPus3p in vitro given the specificity of yeast Pus3p (*22*). The other Ψs in the tRNAs are not modified by either enzyme.

Table 1: Pseudouridine Synthase Activity in Rabbit Reticulocyte Extracts Expressing mPus3p and mPus1p

	mPus3p[a] mol of Ψ/mol of tRNA (SD)[b]		mPus1p mol of Ψ/mol of tRNA (SD)		retic lysate
substrate	observed	expected	observed	expected	mol of Ψ/mol of tRNA (SD)
yeast tRNA^{Phe}	**0.60** (0.041)	1	0.02 (0.001)	0	0.03 (0.002)
yeast tRNA^{Val}	0.13 (0.008)	0	**0.56** (0.033)	1	0.17 (0.005)
human tRNA^{Ser}	**0.55** (0.023)	1	**0.60** (0.036)	1	0.10 (0.007)
human tRNA^{Leu}	**0.67** (0.031)	2	0.04 (0.004)	0	0.04 (0.001)
human tRNA^{Lys3}	**0.52** (0.016)	1	ND[c]		0.10 (0.005)

[a] Aliquots of coupled transcription/translation reactions (Promega TnT Coupled Reticulocyte Lysate system) were added to the substrate RNAs (see Experimental Procedures) and incubated for 30 min at 37 °C. Plasmid DNAs for the expression of mPus3p or mPus1p were used in the transcription/translation reaction; however, in the last column no plasmid DNA was added to the lysate. [b] The observed values shown are the mean and standard deviation of three analyses of one reaction. The mean raw cpm released were corrected for background (no extract or enzyme), and the mol of Ψ/mol of tRNA was calculated from the known amount of substrate added to the reaction and the number of total uridines and Ψ found in the substrate. The expected values are the levels of Ψ in mol of Ψ/mol of tRNA anticipated if the reaction goes to completion. [c] Not determined.

radioactivity found in the spots was quantitated by a phosphoimager, and the results indicate that position 39 is modified by mPus3p (Table 3). As controls, the amounts of Ψ in the 13mer and 4mer from RNase T1 digested tRNA^{Leu} labeled with [^{32}P]CTP were also determined. There was no Ψ in either of these fragments (see Table 3), which indicates that the mPus3p is specific since the 13mer should have no Ψ. In addition, it also indicates that there was no contaminating Ψ 55-specific synthase activity [Pus4; (*16*)], since the 4mer would have Ψ if there was contamination. There was no Ψ found in the tRNAs that were not incubated with mPus3p, as expected (see Table 3).

Table 2: Pseudouridine Synthase Activity in Partially Purified in Vitro Translated mPus3p and Purified Recombinant mPus1p

	mPus3p[a] mol of Ψ/ mol of tRNA (SD)[b]		mPus1p mol of Ψ/ mol of tRNA (SD)	
substrate	observed	expected	observed	expected
yeast $tRNA^{Val}$	0.03 (0.002)	0	**0.40** (0.012)	1
human $tRNA^{Leu}$	**0.32** (0.018)	1	0.01 (0.002)	0

[a] Aliquots of partially purified mPus3p or purified recombinant mPus1p (see Experimental Procedures) were added to the substrate RNAs and incubated for 2 h at 37 °C. [b] As with Table 1, the observed values shown are the mean and standard deviation of three analyses of one reaction. The mean raw cpm released were corrected for background (no extract or enzyme), and the mol of Ψ/mol of tRNA was calculated from the known amount of substrate added to the reaction and the number of total uridines and Ψ found in the substrate. The expected values are the levels of Ψ in mol of Ψ/mol of tRNA anticipated if the reaction goes to completion.

With regard to position 38, Ψ would be detected in the 5mer from $tRNA^{Leu}$ (]^{32}P]UTP-labeled) if mPus3p modified that uridine. In fact, no significant amount of Ψ above background was detected in this fragment when the phosphoimager was used for quantitation. However, when the scan was enhanced, a faint spot for Ψ was seen in this T1 fragment (results not shown).

Table 3: Location of Modifications on tRNA Substrates $tRNA^{Leu}$ and $tRNA^{Ser}$

substrate and fragment[a]	label	expected mol of Ψ/mol of tRNA (position)[b]	observed mol of Ψ/mol of tRNA[c]
$tRNA^{Ser}$ 10mer	CTP	1 (39)	0.24
$tRNA^{Ser}$ (no enzyme)	CTP	2 (39,55)	<0.01
$tRNA^{Leu}$ 5mer	CTP	1 (39)	0.15
$tRNA^{Leu}$ 13mer	CTP	0	0.01
$tRNA^{Leu}$ 4mer	CTP	1 (55)	<0.01
$tRNA^{Leu}$ 5mer	UTP	1 (38)	0.01
$tRNA^{Leu}$ (no enzyme)	CTP	2 (39,55)	<0.01

[a] The substrates were incubated with or without (no enzyme) the purified mPus3p for 1 h at 37 °C, and the RNA was isolated. The RNAs treated with the enzyme were digested with RNase T1, and the product was electrophoresed on a 20% denaturing acrylamide gel. The specific RNase T1 fragments listed were isolated from the gel and digested with RNase T2 and chromatographed on TLC plates in two dimensions. The samples that were not incubated with enzyme were not digested with RNase T1 or gel-purified but were digested directly with RNase T2. [b] The theoretical number and location of pseudouridines are based on all the possible sites that could be labeled given the nucleotide used for labeling the tRNA. In the case of the $tRNA^{Leu}$ 4mer, the uridine at position 55 should not be modified by mPus3p and is a negative control as is the 13mer from this same tRNA. [c] The values that are listed as <0.01 are less than 0.005. The data were obtained using a phosphoimager (BioRad FX) and Quantity One software.

DISCUSSION

The rest of the cloned members of the truA family have been included in Figure 1. As one would expect, the homology between mPus3p and the rest of the members of this pseudouridine synthase family is not as extensive as that between mPus3p and yeast Pus3p. Lecointe et al. (*22*) showed that yeast Pus3p modified positions 38 and 39 in yeast and *E. coli* tRNAs. Unlike *E. coli* PSUI, yeast Pus3p did not modify position 40 on *E. coli* tRNAs with a uridine at position 40, which fits with the fact that yeast tRNAs do not have Ψ at position 40 (*22*). We did not test the activity of mPus3p at position 40, but we assume that it mimics the activity of the yeast enzyme. In support of this assumption is the fact that no sequenced mouse or human tRNAs have a Ψ at position 40 (*52*). In addition, no mouse or human cytoplasmic tRNAs have a thymidine at that position in the genes (*52*).

Yeast Pus1p (*24*) and mPus1p (*17*) were 24% and 22% identical to mPus3p, respectively. The Pus1p enzymes have a different specificity from yeast Pus3p in that they modify positions 27, 28, 34, and 36 in vitro (*17, 24*). Additionally, it has been shown that yeast Pus1p modifies positions 26, 65, and 67 in vivo (*30*). The homology between mPus3p and *E. coli* PSUI (*20*) is the lowest of all the members of the cloned truA family, even though PUSI has essentially the same specificity as mPus3p (*20, 22*). Given this observation, one might expect a higher level of amino acid identity between mPus3p and PSUI.

The regions of homology marked with Roman numerals in Figure 1 (*24, 29*) denote areas of high homology in all the proteins, with the exception of region III. One must keep in mind that in the alignment only those residues with at least three matches among the five members of truA are highlighted. So if the pairs of Pus1p and Pus3p proteins are compared separately, there is a great deal of homology in region III between these sets of proteins, 7 out of 19 and 8 out of 19 for the Pus1p and Pus3p pairs, respectively.

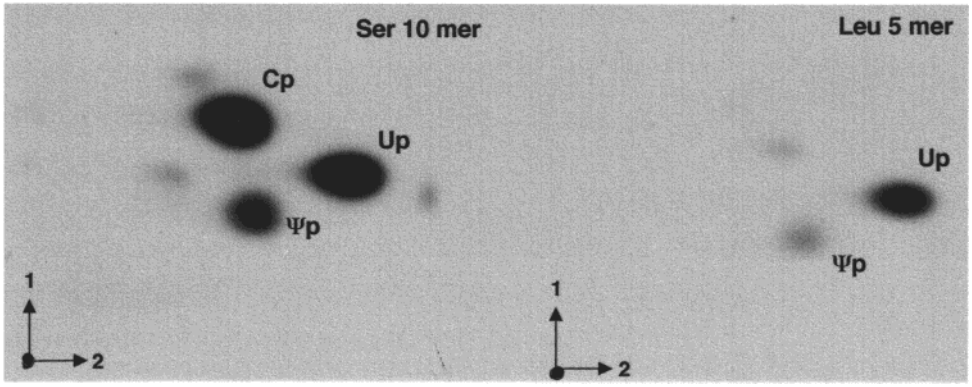

FIGURE 5: mPus3p modifies position 39 on tRNA. Autoradiograms of TLC plates of RNase T1 fragments that were digested with RNase T2 and chromatographed on cellulose plates in the two dimensions indicated (see Experimental Procedures for details). The left side of the panel is the chromatogram of the RNase T1 10mer from human $tRNA^{Ser}$ labeled with [^{32}P]CTP. The right side of the panel is the chromatogram of the RNase T1 5mer from human $tRNA^{Leu}$ labeled with [^{32}P]CTP. The positions of cytodine 3′-monophosphate (Cp), uridine 3′-monophosphate (Up), and pseudouridine 3′-monophosphate (Ψp) are indicated.

mPus3p had a low but detectable activity at position 38 in human $tRNA^{Leu}$. Lecointe et al. (*22*) found that the activity of yeast Pus3p at position 38 was lower than for position 39 but the disparity between the activity at the two positions was not as large as the difference we observed with mPus3p. Pseudouridine was found at position 38 very infrequently in fully sequenced mouse and human tRNAs. There is only 1 mouse example out of 12 tRNAs sequenced and only 3 human examples out of 19 tRNAs sequenced (*52*). This low level of activity of mPus3p for $tRNA^{Leu}$ at position 38 may be due to the fact that this is a recombinant enzyme and that the addition of 21 amino acids to the amino terminus has an effect on the modification at that position. Alternatively it is possible that the concentration of the protein was suboptimal for dimer formation, since it has been shown that the dimer is the active form of *E. coli* PSUI (*53*). Finally, it is possible that this particular substrate is not an optimal one in vitro and in vivo the position may be modified readily (*30*). It is possible that if another tRNA with a uridine at position 38, for instance $tRNA^{Ala}$ (*54*), was used in the assay the activity might be more substantial. Nonetheless, mPus3p does modify $tRNA^{Leu}$ at position 38, albeit very inefficiently.

Yeast Pus3p is sensitive to tRNA tertiary structure since minisubstrates and mutations that affect tertiary structure inhibit formation of Ψ at position 39 (*22*). All yeast tRNAs that have a uridine at position 39 of the gene, have a Ψ at that position, suggesting that the primary sequence is not a determinant for recognition by Pus3p (*22*). In mouse tRNAs that have been sequenced and the modifications determined, 6 out of 12 have a uridine at position 39, and all are converted to Ψ (*52*). With human tRNAs that have been similarly sequenced, 10 out of 19 have a uridine at position 39, and again all are converted to Ψ (*52*).

mPsu3p is a member of the truA family of Ψ synthases, and it has the conserved amino acids known or assumed to be critical for the function of the enzyme. The aspartate that is part of the highly conserved RTDKGV sequence in region II of Figure 1 has been shown to be essential in several Ψ synthases (*33*, *34*, *55*, *56*). The Asp residue is currently thought to be directly involved in catalysis (*53*, *57*, *58*). Since *E. coli* PSUI was sensitive to thiol reagents, it was suggested that cysteines would play a role in the mechanism of Ψ formation (*20*). Subsequently, it was shown that the mutation of the cysteines to other amino acids had no effect on catalysis (*59*, *60*). In addition, no cysteine is conserved among the truA family members (see Figure 1). Although the identity between mPus3p and any of the other members of the truA family besides yeast Pus3p falls off quickly, there is still significant homology in most of the regions designated in Figure 1. Many of these regions contain residues that are near the critical Asp residue in the crystal structure of *E. coli* PSUI (*53*).

The level of mPus3p mRNA is significantly higher in RAW264.7 cells than in either of the mouse tissues tested. This difference may be due to the fact that the cells in culture are rapidly proliferating and there is a greater requirement for protein synthesis and synthesis of tRNAs that require modification. To understand the importance of this result, the relative levels of expression of the mRNA for mPus3p will be determined for several other cell lines and an expanded number of tissues.

Lecointe et al. (*22*) reported a slow growth phenotype in *pus3*- yeast strains, and in fact the original name for this gene was *deg1*, denoting depressed growth (*61*). Likewise, when the gene for PSUI was deleted from *E. coli*, the bacteria exhibit an extreme requirement for uracil in the growth media (*32*). The loss of Ψ near the base of the anticodon stem will affect the stability of the tRNA in this region (*5*, *8*) with possible effects on the efficiency of translation. Since there is an effect on the growth of prokaryotes and yeast when the activity modifying these positions is deleted, what might be the effect of the targeted disruption of mPus3 in mice? Future experiments should provide an answer.

ACKNOWLEDGMENT

We thank Dr. Norman Doggett, who introduced us to the EST data bank years ago. In addition, we thank Dr. O. Uhlenbeck for the yeast $tRNA^{Phe}$ clone, Drs. F. Fasiolo and H. Grosjean for the yeast $tRNA^{Val}$ (with Met anticodon UAC) clone, Dr. R. Marquet for the human $tRNA^{Lys3}$ clone, and Dr. H. Gross for the human $tRNA^{Ser}$ and $tRNA^{Leu}$ clones.

REFERENCES

1. Bjork, G. R., Ericson, J. U., Gustafsson, C. E., Hagervall, T. G., Jonsson, Y. H., and Wikstrom, P. M. (1987) *Annu. Rev. Biochem. 56*, 263−287.
2. Ofengand, J., and Fournier, M. J. (1998) in *Modification and Editing of RNA* (Grosjean, H., and Benne, R., Eds.) pp 229−253, ASM Press, Washington, DC.
3. Massenet, S. A., Mougin, A., and Branlant, C. (1998) in *Modification and Editing of RNA* (Grosjean, H., and Benne, R., Eds.) pp 201−227, ASM Press, Washington, DC.
4. Davis, F. F., and Allen, F. W. (1957) *J. Biol. Chem. 227*, 907−915.
5. Griffey, R. H., Davis, D., Yamaizumi, Z., Nishimura, S., Bax, A., Hawkins, B., and Poulter, C. D. (1985) *J. Biol. Chem. 260*, 9734−9741.
6. Davis, D. R. (1995) *Nucleic Acids Res. 23*, 5020−5026.
7. Davis, D. R., Veltri, C. A., and Nielsen, L. (1998) *J. Biomol. Struct. Dyn. 15*, 1121−1132.
8. Davis, D. R., and Poulter, C. D. (1991) *Biochemistry 30*, 4223−4231.
9. Durant, P. C., and Davis, D. R. (1999) *J. Mol. Biol. 285*, 115−131.
10. Tomita, K., Ueda, T., and Watanabe, K. (1999) *Nucleic Acids Res. 27*, 1683−1689.
11. Zerfass, K., and Beier, H. (1992) *Nucleic Acids Res. 20*, 5911−5918.
12. Perret, V., Garcia, A., Grosjean, H., Ebel, J. P., Florentz, C., and Giege, R. (1990) *Nature 344*, 787−789.
13. Yu, Y. T., Shu, M. D., and Steitz, J. A. (1998) *EMBO J. 17*, 5783−5795.
14. Johnson, L., and Soll, D. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 943−950.
15. Ansmant, I., Massenet, S., Grosjean, H., Motorin, Y., and Branlant, C. (2000) *Nucleic Acids Res. 28*, 1941−1946.
16. Becker, H. F., Motorin, Y., Planta, R. J., and Grosjean, H. (1997) *Nucleic Acids Res. 25*, 4493−4499.
17. Chen, J., and Patton, J. R. (1999) *RNA 5*, 409−419.
18. Conrad, J., Sun, D., Englund, N., and Ofengand, J. (1998) *J. Biol. Chem. 273*, 18562−18566.
19. Huang, L., Ku, J., Pookanjanatavip, M., Gu, X., Wang, D., Greene, P. J., and Santi, D. V. (1998) *Biochemistry 37*, 15951−15957.
20. Kammen, H. O., Marvel, C. C., Hardy, L., and Penhoet, E. E. (1988) *J. Biol. Chem. 263*, 2255−2263.
21. Lafontaine, D. L. J., Bousquet-Antonelli, C., Henry, Y., Caizergues-Ferrer, M., and Tollervey, D. (1998) *Genes Dev. 12*, 527−537.

22. Lecointe, F., Simos, G., Sauer, A., Hurt, E. C., Motorin, Y., and Grosjean, H. (1998) *J. Biol. Chem. 273*, 1316−1323.
23. Raychaudhuri, S., Conrad, J., Hall, B. G., and Ofengand, J. (1998) *RNA 4*, 1407−1417.
24. Simos, G., Tekotte, H., Grosjean, H., Segref, A., Sharma, K., Tollervey, D., and Hurt, E. C. (1996) *EMBO J. 15*, 2270−2284.
25. Wrzesinski, J., Nurse, K., Bakin, A., Lane, B. G., and Ofengand, J. (1995) *RNA 1*, 437−448.
26. Nurse, K., Wrzesinski, J., Bakin, A., Lane, B. G., and Ofengand, J. (1995) *RNA 1*, 102−112.
27. Wrzesinski, J., Bakin, A., Nurse, K., Lane, B. G., and Ofengand, J. (1995) *Biochemistry 34*, 8904−8913.
28. Niu, L., and Ofengand, J. (1999) *Biochemistry 38*, 629−635.
29. Koonin, E. V. (1996) *Nucleic Acids Res. 24*, 2411−2415.
30. Motorin, Y., Keith, G., Simon, C., Foiret, D., Simos, G., Hurt, E., and Grosjean, H. (1998) *RNA 4*, 856−869.
31. Massenet, S., Motorin, Y., Lafontaine, D. L., Hurt, E. C., Grosjean, H., and Branlant, C. (1999) *Mol. Cell. Biol. 19*, 2142−2154.
32. Tsui, H. C., Arps, P. J., Connolly, D. M., and Winkler, M. E. (1991) *J. Bacteriol. 173*, 7395−7400.
33. Raychaudhuri, S., Niu, L., Conrad, J., Lane, B. G., and Ofengand, J. (1999) *J. Biol. Chem. 274*, 18880−18886.
34. Conrad, J., Niu, L., Rudd, K., Lane, B. G., and Ofengand, J. (1999) *RNA 5*, 751−763.
35. Zebarjadian, Y., King, T., Fournier, M. J., Clarke, L., and Carbon, J. (1999) *Mol. Cell. Biol. 19*, 7461−7472.
36. Lennon, G., Auffray, C., Polymeropoulos, M., and Soares, M. B. (1996) *Genomics 33*, 151−152.
37. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
38. Sampson, J. R., and Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1033−1037.
39. Szweykowska-Kulinska, Z., Senger, B., Keith, G., Fasiolo, F., and Grosjean, H. (1994) *EMBO J. 13*, 4636−4644.
40. Isel, C., Marquet, R., Keith, G., Ehresmann, C., and Ehresmann, B. (1993) *J. Biol. Chem. 268*, 25269−25272.
41. Breitschopf, K., and Gross, H. J. (1994) *EMBO J. 13*, 3166−3169.
42. Wu, X. Q., and Gross, H. J. (1993) *Nucleic Acids Res. 21*, 5589−5594.
43. Mullis, K. B., and Faloona, F. A. (1987) *Methods Enzymol. 155*, 335−350.
44. Church, D. M., and Buckler, A. J. (1999) *Methods Enzymol. 303*, 83−99.
45. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem. 162*, 156−159.
46. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
47. Laemmli, U. K. (1970) *Nature 227*, 680−685.
48. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984) *Nucleic Acids Res. 12*, 7035−7056.
49. Patton, J. R. (1991) *Mol. Cell. Biol. 11*, 5998−6006.
50. Cortese, R., Kammen, H. O., Spengler, S. J., and Ames, B. N. (1974) *J. Biol. Chem. 249*, 1103−1108.
51. Nishimura, S. (1972) *Prog. Nucleic Acid Res. Mol. Biol. 12*, 49−85.
52. Sprinzl, M., Horn, C., Brown, M., Ioudovitch, A., and Steinberg, S. (1998) *Nucleic Acids Res. 26*, 148−153.
53. Foster, P. G., Huang, L., Santi, D. V., and Stroud, R. M. (2000) *Nat. Struct. Biol. 7*, 23−27.
54. Bunn, C. C., and Mathews, M. B. (1987) *Mol. Biol. Med. 4*, 21−36.
55. Huang, L., Pookanjanatavip, M., Gu, X., and Santi, D. V. (1998) *Biochemistry 37*, 344−351.
56. Ramamurthy, V., Swann, S. L., Paulson, J. L., Spedaliere, C. J., and Mueller, E. G. (1999) *J. Biol. Chem. 274*, 22225−22230.
57. Gu, X., Liu, Y., and Santi, D. V. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 14270−14275.
58. Lee, T.-S., and Kollman, P. A. (1999) *J. Am. Chem. Soc. 121*, 9928−9931.
59. Zhao, X., and Horne, D. A. (1997) *J. Biol. Chem. 272*, 1950−1955.
60. Ramamurthy, V., Swann, S. L., Spedaliere, C. J., and Mueller, E. G. (1999) *Biochemistry 38*, 13106−13111.
61. Carbone, M. L., Solinas, M., Sora, S., and Panzeri, L. (1991) *Curr. Genet. 19*, 1−8.

BI001109M