Caenorhabditis elegans pseudouridine synthase 1 activity *in vivo*: tRNA is a substrate, but not U2 small nuclear RNA[1]

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The formation of pseudouridine (Ψ) from uridine is post-transcriptional and catalysed by pseudouridine synthases, several of which have been characterized from eukaryotes. Pseudouridine synthase 1 (Pus1p) has been well characterized from yeast and mice. In yeast, Pus1p has been shown to have dual substrate specificity, modifying uridines in tRNAs and at position 44 in U2 small nuclear RNA (U2 snRNA). In order to study the *in vivo* activity of a metazoan Pus1p, a knockout of the gene coding for the homologue of Pus1p in *Caenorhabditis elegans* was obtained. The deletion encompasses the first two putative exons and includes the essential aspartate that is required for activity in truA pseudouridine synthases. The locations of most modified nucleotides on small RNAs in *C. elegans* are not known, and the positions of Ψ were determined on four tRNAs and U2 snRNA. The uridine at position 27 of $tRNA^{Val}$ (AAC), a putative Pus1p-modification site, was converted into Ψ in the wild-type worms, but the $tRNA^{Val}$ (AAC) from mutant worms lacked the modification. Ψ formation at positions 13, 32, 38 and 39, all of which should be modified by other pseudouridine synthases, was not affected by the loss of Pus1p. The absence of Pus1p in *C. elegans* had no effect on the modification of U2 snRNA *in vivo*, even though worm U2 snRNA has a Ψ at position 45 (the equivalent of yeast U2 snRNA position 44) and at four other positions. This result was unexpected, given the known dual specificity of yeast Pus1p.

Key words: knockout, primer extension, truA.

INTRODUCTION

Pseudouridine (Ψ) is an abundant post-transcriptional modification found in stable RNA species, including tRNA, rRNA and small nuclear RNAs (snRNAs), and the sites of modification on these RNAs are highly conserved in eukaryotes [1–5]. Many pseudouridine synthases have been cloned and characterized and one, pseudouridine synthase 1 (Pus1p), has been studied extensively in yeast and mice [6–9]. These two proteins, members of the truA family of pseudouridine synthases, have a high degree of homology that is characterized by stretches of near-perfect identity. Yeast and mouse Pus1p have been shown to modify positions 27/28 of many tRNAs and the anticodons of some intron-containing tRNAs *in vitro* [6,8]. In addition to modifying tRNA, yeast Pus1p is responsible for the modification of position 44 on U2 snRNA, an RNA involved in the splicing of pre-messenger RNA [10]. The *in vivo* activity of yeast Pus1p also includes the modification of uridines at positions 26, 65 and 67 in tRNAs [7].

Yeast Pus1p does not require cofactors, such as guide RNAs, to modify tRNAs or U2 snRNA in specific positions [6,7,10]. Likewise there is no requirement for RNA cofactors in the modification of tRNA by mouse Pus1p [8]. This is in contrast with the formation of Ψ on rRNA in eukaryotes, which requires small nucleolar RNAs (snoRNAs) in addition to a pseudouridine synthase [11,12]. Interestingly, the formation of Ψ in snRNAs includes site-specific modification, as mentioned above for yeast U2 snRNA, and snoRNA-dependent modification for U5 and U6 snRNAs in mammals [13,14]. In *Xenopus*, the pseudouridylation of two positions in the branch-point-recognition region of U2 snRNA requires an snoRNA cofactor [15].

Because of the additional imino proton on the base, Ψ is potentially more versatile in its hydrogen-bonding interactions than uridine [16]. The presence of Ψ has been shown to stabilize base stacking in oligonucleotides [17] and to contribute to the stability of hydrogen bonding [18–20]. Ψ appears to be necessary for the alternative reading of codons [21,22] and to prevent mischarging of the tRNA [23]. The presence of Ψ is necessary for the function of U2 snRNA in the splicing of pre-messenger RNA in *Xenopus* oocytes [24]. In addition, U2 small nuclear ribonucleoprotein particles assembled *in vitro* from U2 snRNA isolated from HeLa cells reconstituted a U2 small nuclear ribonucleoprotein particle-depleted pre-messenger RNA splicing assay but U2 snRNA synthesized *in vitro*, which lacked modifications, does not reconstitute the depleted splicing extract [25].

When genes for Pus1p and pseudouridine synthase 4 (Pus4p) were deleted separately in yeast, there was no effect on growth [6,26], but if the disruption of the genes for Pus1p and Pus4p was combined then the result was lethal [27]. Pus4p modifies tRNA at position 55, where nearly all tRNAs have a Ψ [1,2]. It appears that the loss of these two enzymes affects the transport of tRNA from the nucleus [27]. Additionally, when the deletion of the *PUS1* gene in yeast was combined with the deletion of *LOS1* (a gene involved with tRNA maturation that when deleted alone is not lethal), the combination was lethal [6]. Recently it has been shown that the loss of truB, the Pus4p homologue in *Escherichia coli*, results in sensitivity to temperature stress [28].

Caenorhabditis elegans is a model where traditional genetic manipulations and mutagenesis can be applied to a multicellular organism to study the developmental consequences of gene

Abbreviations used: Ψ, pseudouridine; Pus1p, pseudouridine synthase 1; Pus3p, pseudouridine synthase 3; Pus4p, pseudouridine synthase 4; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; CMCT, 1-cyclohexyl-3(2-morpholinoethyl) carbodi-imide metho-*p*-toluenesulphonate.

[1] This work is dedicated to the memory of Scott T. Worth, who greatly influenced our decisions to become scientists.

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products [29]. A mutant worm with a deletion in the gene that codes for the Pus1p homologue in *C. elegans* was isolated and the positions equivalent to those residues in tRNA modified by Pus1p characterized from yeast and mouse were not modified in the mutant worms. Unexpectedly, the modification of U2 snRNA at position 45 was unaffected by the loss of Pus1p, which means that, unlike yeast, Pus1p is not required for the modification of U2 snRNA at that position in worms.

EXPERIMENTAL

Materials

The *C. elegans* gene that codes for the homologue of Pus1p (W06H3.2) was identified by a BLAST search on WormBase (http://www.wormbase.org), and the cDNA clone containing the full-length coding region of the *C. elegans* homologue for Pus1p (yk174d5) was obtained from Y. Kohara (National Institute of Genetics, Mishima, Japan). The knockout of the *C. elegans* gene that codes for the homologue of Pus1p [strain VC110, W06H3.2 (gk38)] was provided by the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia, Vancouver, BC, Canada. This strain was outcrossed to the wild-type (N2 [29]) five times.

Constructs

The gene for $tRNA^{Ala}$ (AGC) was amplified from *C. elegans* genomic DNA using the PCR [30,31] and the following primers: 5′-GGGGGTATAGCTCAGTGG-3′ and 5′-GGAGGTATGGGGAATTGA-3′. The resulting fragment was inserted into pGEMT (Promega) and the recombinant plasmid DNA was used in the Ψ assay as a sequencing template (see below). The entire cDNA coding for the *C. elegans* homologue of Pus1p was amplified using PCR, yk174d5 DNA, and the following primers: 5′-GGATCCATGACAAAAACCGTTGAGGA-3′ and 5′-GCGGCCGCTTATCCAGACGACGTCGCTGC-3′. The PCR products were first inserted into pGEMT and sequenced [32].

Growth of *C. elegans*

The worms were grown at 20 °C, unless otherwise indicated, on nematode growth medium plates seeded with *E. coli* strain OP50 [33,34]. The outcrossing of the VC110 mutant worms was accomplished using single worm PCR [35,36] and the following primers: sense, 5′-GCCAAGGTTTCCGGGGAAAG-3′; antisense, 5′-GCCCGGCATCTCTGTAATAG-3′.

Assay for Ψ in small RNAs from worms

The method for the mapping of Ψ residues has been described previously [37,38]. Total RNA from N2 (wild type) and VC110 worms was isolated as described [34] and the RNA treated with two concentrations of CMCT [1-cyclohexyl-3(2-morpholinoethyl) carbodi-imide metho-*p*-toluenesulphonate; Aldrich] as described [37]. An aliquot of each type of RNA was also left untreated for comparison. After treatment with sodium carbonate to remove all the adducts, but not those attached to Ψ residues, the RNA samples were used as templates for reverse transcription using avian myeloblastosis virus reverse transcriptase (Promega) and ^{32}P-end-labelled primers [38,39]. The primers used were as follows: $tRNA^{Ala}$ (AGC), 5′-GGAGGTATGGGGAATTGA-3′; $tRNA^{Leu}$ (AAG), 5′-GAGAGTGGGATTCGAACC-3′; $tRNA^{Val}$ (AAC), 5′-TGATCTCGGGCGGGCTCGAA-3′; U2 snRNA, 5′-CCGAGTCTTCCCTAGGTTCC-3′. The *fmole*® Sequencing kit (Promega) was used to generate the sequencing lanes for all gels [32,38]. The pGEMT-$tRNA^{Ala}$ clone was used as the template for sequencing $tRNA^{Ala}$ and PCR fragments generated from *C. elegans* genomic DNA were used for the sequencing of the other RNAs. The forward primers used to generate those fragments were: $tRNA^{Leu}$ (AAG), 5′-GAGAGATGGCCGAGCGGT-3′; $tRNA^{Val}$ (AAC), 5′-GGTCTCGTGGTGTAGTGG-3′; U2 snRNA, 5′-CGCTTCTTCGGCTTATTAG-3′. The reverse primers used to generate the fragments are listed above in the section on primer extension.

Southern blotting

Genomic DNA (2 μg) from N2 and VC110 worms, isolated as described in [34], was digested with *Eco*RI, *Hae*III and *Hin*dIII and electrophoresed on a 1 % agarose gel. The gel was transferred to nitrocellulose and the blot probed with ^{32}P-labelled *C. elegans* Pus1 sense RNA transcribed with SP6 RNA polymerase from pGEMT-*C. elegans* Pus1 DNA digested with *Nco*I [40].

RESULTS

A BLAST search using either yeast [6] or mouse [8] Pus1p identified a gene in WormBase, W06H3.2 (on chromosome V), with a predicted open reading frame that had 21 % identity with the yeast protein and 29 % identity with the mouse protein (Figure 1). Using microarray technology, Hill et al. [41] have shown that this gene is expressed in all stages of development and into adulthood. In the alignment, there are stretches of perfect or near-perfect identity, and one such stretch in W06H3.2 is between residues 85 and 95. This portion of the *C. elegans* homologue contains the aspartate (residue 92) that is known to be at the catalytic centre of the *E. coli* protein PSUI (truA) and is required for activity [42–44]. Based on homology, this gene is most probably the worm homologue of Pus1p. It is interesting to note that with the Pus1p homologues, the mouse and worm versions are approximately the same size, whereas yeast Pus1p contains an insert of approx. 80 amino acids that begins at residue 204. This, along with the additional residues found at the N-terminus, results in a considerably larger size for the yeast protein [6].

The expression of the *C. elegans* homologue in bacteria or *in vitro* transcription/translation systems did not result in an active protein (results not shown). Therefore, in order to study the specificity of *C. elegans* Pus1p, we obtained a knockout of the W06H3.2 gene [strain VC110, W06H3.2 (gk38)] from the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia. This mutant worm provides the basis for the assay of the activity of Pus1p in *C. elegans*. The tRNAs and U2 snRNA from the mutant worms should be missing Ψ at specific positions when compared with RNAs from wild-type worms. The deletion in the W06H3.2 gene is 437 bp, includes 57 bp 5′ of the putative AUG codon and extends 28 bp into the second intron. Therefore the first two exons, which code for the first 100 amino acids of the predicted protein, would be missing from any RNA that might be transcribed from this mutant gene. Since the aspartate that has been shown to be required for activity is located at position 92 in the *C. elegans* Pus1p homologue (Figure 1), even if a protein is made from the mRNA, it would not be active [42–44]. The strain was outcrossed five times to N2 males. A Southern blot of genomic DNA was consistent with the small deletion, and there was no gross rearrangement of the DNA in the vicinity of the gene in the mutant worms (results not shown).

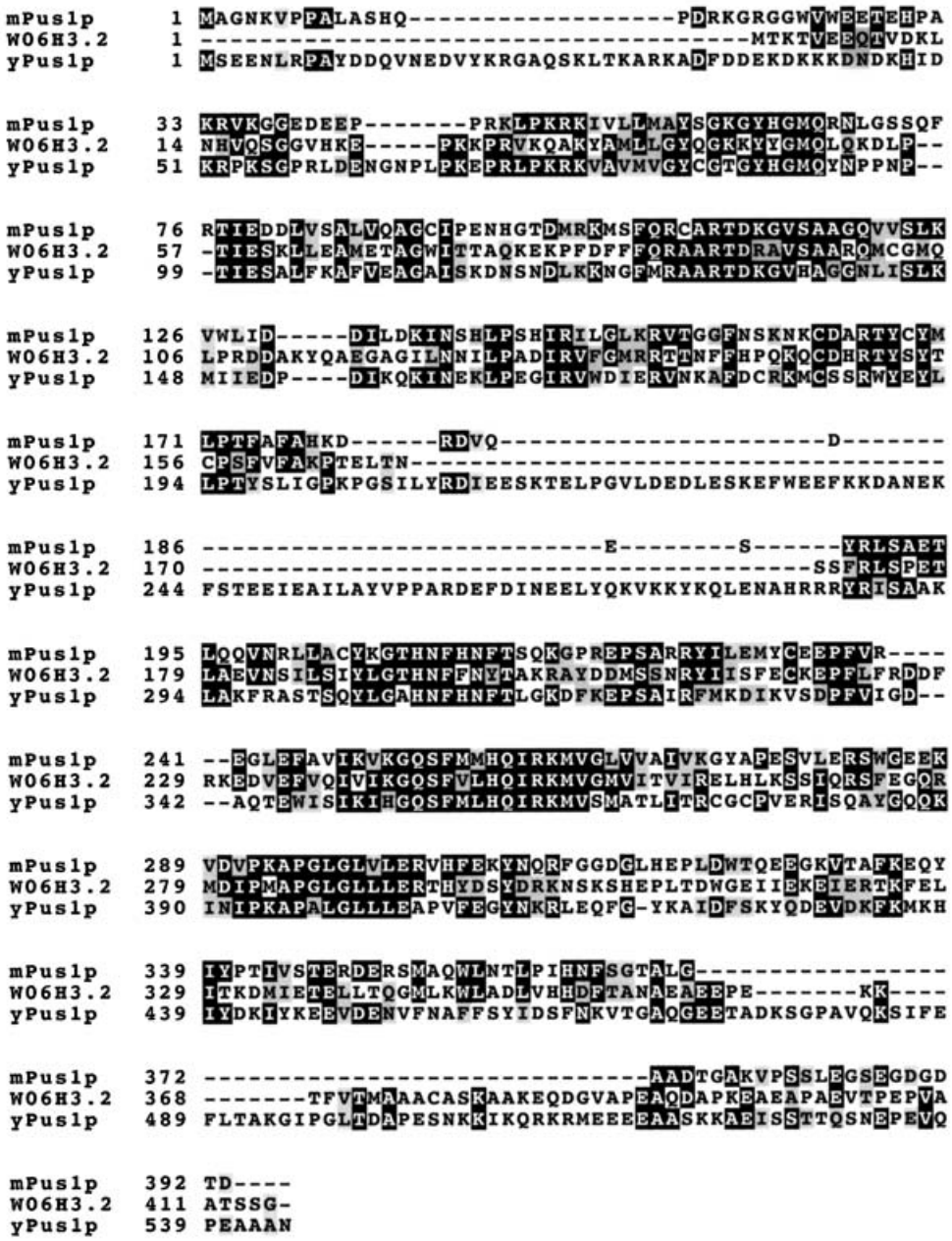

Figure 1 Homology of Pus1p from yeast (yPus1p), mouse (mPus1p) and *C. elegans* (W06H3.2)

Black-boxed residues denote identity and grey-boxed residues denote conservative replacement. The *C. elegans* homologue is 21 % identical with yPus1p and 29 % identical with mPus1p.

The mutant worms are superficially wild type and this allows for the isolation of sufficient material to assay for Pus1p activity. When grown at 20 °C, there is no significant difference between N2 and VC110 worms in terms of fecundity, size or anatomy. The male sexual apparatus in the mutant worms appears the same as in the wild-type worms and when VC110 hermaphrodites are mated with mutant males, 46 $\pm$ 5 % (mean $\pm$ S.D.) of the progeny are males, as expected. Embryogenesis appears to take the same length of time with both types of worm. However, there is a slight difference in how quickly the mutant hermaphrodites develop. The wild-type N2 worms start laying eggs 2–3 h before the VC110 worms. The life spans for the N2 and VC110 worms are essentially the same.

The modification state of several small RNAs from N2 and VC110 worms was determined. This was accomplished using a primer-extension method that involves the chemical modification of total RNA, which after further manipulation leaves only the Ψ residues chemically modified [37,38]. End-labelled primers specific for particular RNAs are used in reverse-transcription reactions on total RNA, and the modified residues are strong stops to the enzyme that are not found in untreated RNA controls. Strong stops will be observed on the autoradiographs of the gels at the nucleotide just before the modified residue [37,38].

The modification state of most *C. elegans* tRNAs is not known, but many tRNA genes have been identified in various databases [45,46]. Several of those tRNA genes, which have uridines in

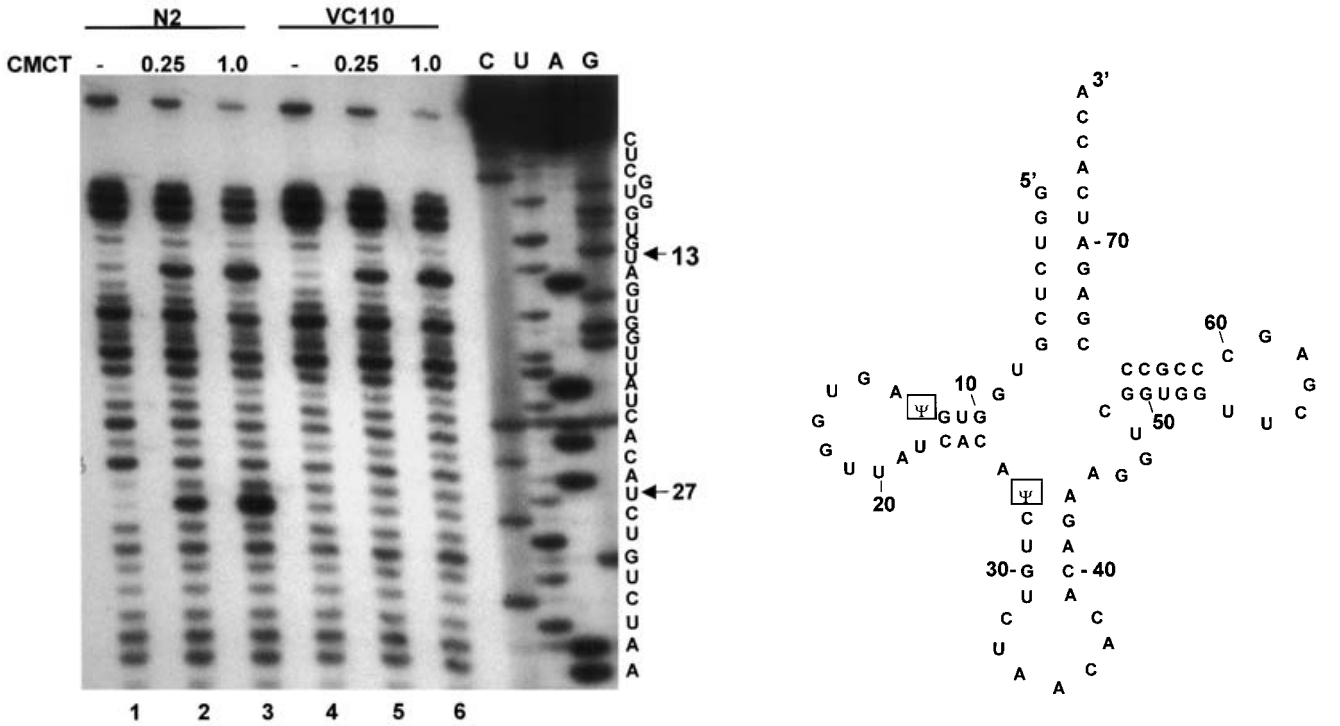

Figure 2 Assay for Ψ in *C. elegans* tRNA^{Val} (AAC)

Left-hand panel: the primer-extension assay described in the Experimental section was used to determine the modification state of tRNA^{Val} (AAC) in total RNA isolated from N2 (wild-type) and VC110 (mutant) worms. The RNAs were treated with two different concentrations of CMCT and the products of the primer-extension reactions using an end-labelled primer were electrophoresed on a 10 % polyacrylamide/8.3 M urea denaturing gel. Chain-termination sequencing using the same primer and a tRNA^{Val} (AAC) PCR template is shown on the right. Two positions that are modified (Ψ) are shown with arrows on the right. The extension shows a strong stop just before the modification. Those two positions are indicated on a secondary-structure diagram for tRNA^{Val} (AAC) in the right-hand panel.

positions known to be modified by pseudouridine synthases [6–8], were assayed. For example, tRNA^{Val} (AAC) has a uridine at position 27 and should be a potential substrate for Pus1p. Other residues that might be modified to Ψ are 13 and 55 in this tRNA, but Pus1p is not expected to be responsible for those modifications. The results of the analysis are shown in Figure 2 (left-hand panel) and it can be seen that at position 13 there is Ψ in the tRNA from both N2 and VC110 worms. There is a strong stop to reverse transcriptase just prior to position 13 in the CMCT-treated lanes, but not in the untreated lanes (see lanes 1–6 in Figure 2, left-hand panel). This result was expected given that another, as yet unidentified, pseudouridine synthase is responsible for the modification of uridine at position 13 [7]. However, at position 27, a residue that should be modified by Pus1p [7,8], there are strong stops to reverse transcriptase only in the RNAs from N2 worms (Figure 2, left-hand panel, lanes 2 and 3). The tRNA^{Val} from VC110 has no strong stop at position 27 (Figure 2, left-hand panel, lanes 5 and 6) and therefore no Ψ at this position, as we would have expected given that the enzyme is missing in these worms. The two Ψs that were mapped in the N2 worms are shown boxed in the tRNA^{Val} secondary-structure diagram in Figure 2 (right-hand panel).

A second tRNA to assay, tRNA^{Ala} (AGC), was chosen because it had a uridine at position 38 and it is potentially a substrate for pseudouridine synthase 3 (Pus3p), and so the loss of Pus1p should have no effect on the modification at this position. Pus3p from yeast and mouse modifies positions 38 and 39 in the anticodon stem-loop of tRNAs [47,48]. In Figure 3 (left-hand panel) it is easy to see that just prior to position 38 there are strong stops to reverse transcriptase in the RNA samples from both N2 and VC110 worms (Figure 3, left-hand panel, lanes 2, 3, 5 and 6). The origin of this double band is not known, but since there is only one uridine in the sequence near this position and a distance larger than the doublet separates the nucleotides, there is only one Ψ at this position. These stops do not appear in the RNA samples that were not treated with CMCT (lanes 1 and 4). The position of the mapped modification is shown on the tRNA^{Ala} secondary-structure diagram in Figure 3 (right-hand panel). Other tRNAs tested were tRNA^{Leu} (AAG) and tRNA^{Ser} (UGA), and the results indicated that those modified uridines at positions 32 and 39 were not affected by the loss of Pus1p (results not shown). These are the results expected, since Pus1p is not known to modify either of these two positions *in vivo* [7].

It has been shown both *in vitro* and *in vivo* that *Saccharomyces cerevisiae* Pus1p is responsible for the modification of U2 snRNA at position 44 [10]. U2 snRNA from *S. cerevisiae* has two other positions modified (35 and 42), but other pseudouridine synthases are responsible for those modifications [10,48a]. Given the degree of homology between yeast and *C. elegans* Pus1p and the conservation of the U2 snRNA sequences in the two organisms, it was assumed that *C. elegans* U2 snRNA would be modified by Pus1p. The modification state of *C. elegans* U2 snRNA was tested in total RNA derived from N2 and VC110 worms and the results are shown in Figure 4 (top panel). Three strong stops to reverse transcriptase and therefore Ψ modifications are seen easily in the RNA from both N2 and VC110 worms in the lighter exposure of the gel (Figure 4, top panel, lanes 2, 4 and 5). Positions 45, 46 and 56 are modified and those residues are marked on the secondary-structure diagram of the 5′ end of *C. elegans* U2 snRNA shown in Figure 4 (bottom right panel). At high CMCT concentrations

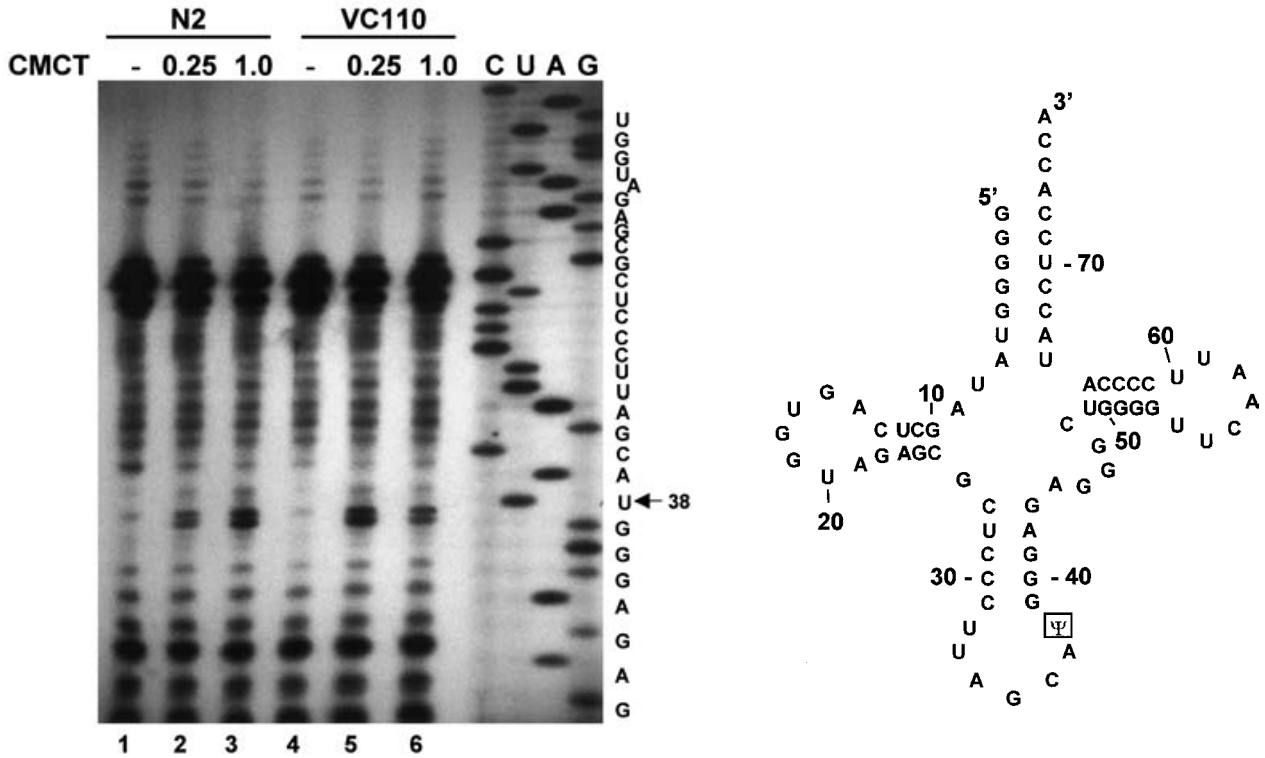

Figure 3 Assay for Ψ in *C. elegans* tRNA^{Ala} (AGC)

Left-hand panel: the primer-extension assay described in the Experimental section was used to determine the modification state of tRNA^{Ala} (AGC) in total RNA isolated from N2 (wild-type) and VC110 (mutant) worms. The RNAs were treated and electrophoresed as described in the legend for Figure 2. Chain-termination sequencing using the same primer and plasmid DNA that contains the tRNA^{Ala} (AGC) gene is shown on the right. The position of U^{38} is shown with an arrow on the right. Note that the extension shows a strong stop just before the modification. The position that was mapped is indicated on a secondary-structure diagram for *C. elegans* tRNA^{Ala} (AGC) in the right-hand panel. Note that *C. elegans* tRNA^{Ala} has a shortened D-loop and is missing a nucleotide at position 17.

a strong stop is also seen before position 48 (see lanes 2 and 5, Figure 4, top panel). Position 45 in worm U2 snRNA corresponds to position 44 in yeast U2 snRNA [49] and since that position is modified in both N2 and VC110 worms, the *C. elegans* Pus1p is not required for the modification of that particular uridine. This portion of U2 snRNA is very highly conserved, since it interacts with the lariat branch point during pre-mRNA splicing and so it was straightforward to determine which positions in *C. elegans* U2 snRNA correspond to those in the yeast homologue. With longer exposure of the same gel (Figure 4, bottom left panel), an additional stop to reverse transcriptase can be seen just prior to position 39 in the RNAs treated with CMCT from either N2 or VC110 worms (Figure 4, bottom left panel, lanes 2, 4 and 5). This position is indicated with an arrow in the panel and the structural diagram of the 5′ end of U2 snRNA (Figure 4, bottom right panel). There may be partial modification at positions 39 and 48, since the intensity of the bands at those positions are both less than the other bands at positions 45, 46 and 56. There are three Ψ in yeast U2 snRNA, at positions 35, 42 and 44, and yeast Pus1p only modifies position 44 [10]. There are at least five Ψ residues in this region of *C. elegans* U2 snRNA, and in worms the Pus1p homologue is not required for the modification of any of those positions.

DISCUSSION

Pus1p from yeast has been shown to modify *S. cerevisiae* U2 snRNA both *in vivo* and *in vitro* at position 44 [10]. It was fully expected that the *C. elegans* homologue of Pus1p would also modify at least the equivalent position in worm U2 snRNA. But this was not the case; the equivalent position in worm U2 snRNA, position 45, is pseudouridylated even when Pus1p is missing in the VC110 mutants. What could be the reasons for this unexpected result? It is possible that, in metazoans in general, Pus1p is not responsible for this modification and a different pseudouridine synthase carries out the modification at position 45. This process might employ guide RNAs, as in the case of the modification of rRNA in eukaryotes, which requires snoRNA cofactors to act as guides for specific pseudouridylation [11,12]. This seems the most parsimonious explanation of the results in light of the fact that the modification of certain residues in U5 and U6 snRNAs in mammals [14,50] and in the branch-point-recognition region of U2 snRNA from *Xenopus* [15] have been shown to require snoRNAs. The mode used to modify *C. elegans* U2 snRNA may have more in common with that used by the more complex eukaryotes than with the reliance on Pus1p seen in yeast. If this is the case, the pseudouridine synthase that is responsible for the modification of U2 snRNA in worms may be a protein that is homologous with Cbf5p or dyskerin, the proteins from yeast and human respectively, that are responsible for the modification of rRNA and require snoRNA cofactors [51,52]. It is possible that this site (Ψ-45) is modified by both Pus1p and the snoRNA-guided pseudouridylation systems. When Pus1p is absent, the modification at this position is still seen, since the redundant system is still functional. Zhao et al. [15] have suggested redundant pseudouridylation mechanisms in the modification of position 34 in U2 snRNA in *Xenopus*.

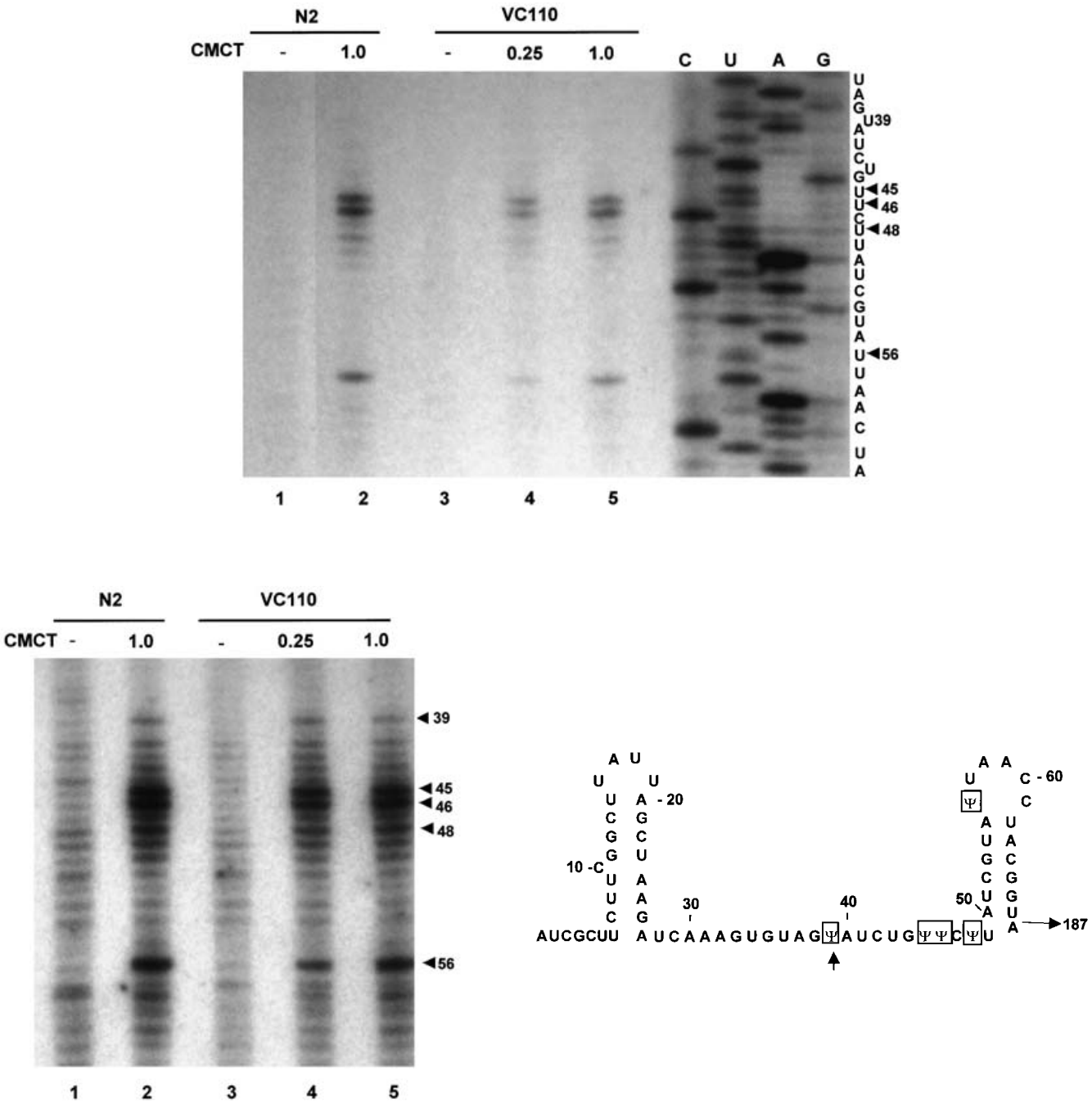

Figure 4 Assay for Ψ in *C. elegans* U2 snRNA

Top panel: the primer-extension assay described in the Experimental section was used to determine the modification state of U2 snRNA in total RNA isolated from N2 (wild-type) and VC110 (mutant) worms. The RNAs were treated and electrophoresed as described in the legend for Figure 2. Chain-termination sequencing using the same primer and a U2 snRNA PCR template made from *C. elegans* genomic DNA is shown on the right. Four positions that are modified (Ψ) are shown with arrows on the right of the panel. The extension shows a strong stop just before the modification. Those four positions are indicated on a secondary-structure diagram (bottom right panel) of the 5′ end of *C. elegans* U2 snRNA. A longer exposure of the gel to film (bottom left panel) revealed an additional site of modification at position 39 (arrow in the bottom left panel) and is indicated in the sequence in the top panel. It is also indicated on the secondary-structure diagram with an arrow.

Alternatively, in *C. elegans* there may be more than one site-specific pseudouridine synthase that can convert the uridine at position 45 in U2 snRNA. When Pus1p is absent, as in the case of the VC110 worms, the backup synthase would modify the site. This putative synthase would not require cofactors such as snoRNAs. This possibility seems unlikely, since site-specific pseudouridine synthases such as Pus1p, Pus3p and Pus4p are highly specific and there is no evidence thus far for overlapping specificities [7]. Interestingly, the yeast Pus1p protein contains an additional stretch of ≈80 amino acids, a stretch which has no homology with metazoan Pus1p homologues (see Figure 1). Perhaps this additional motif allows the yeast enzyme to recognize U2 snRNA.

The sequence of U2 snRNA is highly conserved and the number of uridines modified to Ψ in this spliceosomal snRNA can be roughly correlated with the complexity of the organism.

S. cerevisiae has three Ψs in U2 snRNA at positions 35, 42 and 44 [10], but *Schizosaccharomyces pombe* U2 snRNA has the equivalent of those three plus an additional three at *S. pombe* U2 snRNA positions 37, 44 and 58 [53]. Human U2 snRNA has 12 Ψs, but in this region it has eight of the modifications, at positions 34 (*S. cerevisiae* position 35), 37, 39, 41 (*S. cerevisiae* position 42), 43 (*S. cerevisiae* position 44), 44, 54 and 58 [54]. *C. elegans* has at least five Ψs in this region of U2 snRNA, at positions 39 (*S. pombe* and human position 37), 45 (*S. cerevisiae* position 44 and *S. pombe* and human positions 43), 46 (*S. pombe* and human positions 44), 48 and finally at position 56 (equivalent to position 54 in human U2 snRNA). *C. elegans* U2 snRNA does not have a uridine at the position equivalent to position 59 in *S. pombe* and 58 in human U2 snRNA, and therefore it cannot be modified to Ψ. So the pattern of modification of *C. elegans* U2 snRNA in this region is unique in the sense that it has a Ψ at position 48, but it also has Ψs at sites that are equivalent to the modified residues in other sequenced U2 snRNAs. The full 5′ end of *C. elegans* U2 snRNA was not analysed for modifications and it is possible that additional Ψs will be found upon more detailed mapping. But again none of the Ψs mapped in the U2 snRNA isolated from worms required Pus1p.

This paper describes the first report of the elimination of specific Ψs in the RNAs of a metazoan. Given the high conservation of the sites of Ψ modification in tRNAs and the expression of the Pus1p homologue at all stages of development [41], it was surprising that the loss of this enzyme had little effect on the mutant worm. This is a highly conserved enzyme [6,8] that converts specific uridines in many tRNAs into a modified nucleotide that is known to strengthen base-pairing and stabilize base-stacking [16,17,20]. The homologue identified in *C. elegans* is the best match for Pus1p in the *C. elegans* genome. The overall homology for W06H3.2 with yeast and mouse Pus1p is significant and regions that are highly conserved in the truA family are also conserved in this worm homologue [55]. In addition, the results of the mapping of Ψ residues on the worm tRNAs show a loss of Pus1p activity in $tRNA^{Val}$ with the VC110 mutants. The *C. elegans* homologue is likely to modify the same sites as yeast [7] and mouse [8] Pus1p, but additional mapping studies will be necessary to verify that conclusion.

C. elegans develops so quickly that even minor perturbations of cellular processes might be expected to have an impact on growth and development. In this report, the absence of Pus1p had no profound effect on the worms. Previous experiments in yeast have shown that disruption of the *PUS1* gene had no phenotype [6], but recently it was shown that if the disruption of the genes for Pus1p and Pus4p (pseudouridine-55 synthase) were combined in yeast, the result was lethal [27]. The authors showed that the transport of at least one tRNA from the nucleus is inhibited when Pus1p is missing. Therefore, the modification of tRNAs by Pus1p becomes essential if other aspects of tRNA metabolism are affected, as in the case of the additional loss of Pus4p [27]. With all the isoacceptor tRNAs that exist in cells, a phenotype occurs only when the available pool of tRNAs is diminished. Unfortunately, a homologue of yeast or mammalian Pus4p could not be identified in the worm genome using various BLAST tools and several databases. The search for the functional equivalent of Pus4p in the worm is an immediate concern, since a double knockout of the genes coding for Pus1p and Pus4p activity is an obvious experiment, given the results in yeast. It is likely that worms do modify position 55 in tRNAs, since that residue is converted into Ψ in nearly all prokaryotic and eukaryotic cytoplasmic tRNAs that have been sequenced. In addition, all *C. elegans* cytoplasmic tRNA genes have a uridine at position 55 [45,46].

The usefulness of a model system such as *C. elegans* for the study of the specificity of these highly conserved enzymes has been shown in this study. In addition to the characterization of additional modification enzymes *in vivo* in terms of substrate specificity and cofactor requirements, the effects of the loss of other pseudouridine synthases will be the focus of future studies.

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REFERENCES

1 Auffinger, P. and Westhof, E. (1998) Appendix 5: Location and distribution of modified nucleotides in tRNA. In Modification and Editing of RNA (Grosjean, H. and Benne, R., eds.), pp. 569–576, ASM Press, Washington DC
2 Bjork, G. R., Ericson, J. U., Gustafsson, C. E., Hagervall, T. G., Jonsson, Y. H. and Wikstrom, P. M. (1987) Transfer RNA modification. Annu. Rev. Biochem. **56**, 263–287
3 Johnson, L. and Soll, D. (1970) *In vitro* biosynthesis of pseudouridine at the polynucleotide level by an enzyme extract from *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **67**, 943–950
4 Massenet, S. A., Mougin, A. and Branlant, C. (1998) Posttranscriptional modifications of the U small nuclear RNAs. In Modification and Editing of RNA (Grosjean, H. and Benne, R., eds.), pp. 201–227, ASM Press, Washington DC
5 Ofengand, J. and Fournier, M. J. (1998) The pseudouridine residues of rRNA: Number, location, biosynthesis, and function. In Modification and Editing of RNA (Grosjean, H. and Benne, R., eds.), pp. 229–253, ASM Press, Washington, D.C
6 Simos, G., Tekotte, H., Grosjean, H., Segref, A., Sharma, K., Tollervey, D. and Hurt, E. C. (1996) Nuclear pore proteins are involved in the biogenesis of functional tRNA. EMBO J. **15**, 2270–2284
7 Motorin, Y., Keith, G., Simon, C., Foiret, D., Simos, G., Hurt, E. and Grosjean, H. (1998) The yeast tRNA : pseudouridine synthase Pus1p displays a multisite substrate specificity. RNA **4**, 856–869
8 Chen, J. and Patton, J. R. (1999) Cloning and characterization of a mammalian pseudouridine synthase. RNA **5**, 409–419
9 Chen, J. and Patton, J. R. (2000) Mouse pseudouridine synthase 1: gene structure and alternative splicing of pre-mRNA. Biochem. J. **352**, 465–473
10 Massenet, S., Motorin, Y., Lafontaine, D. L., Hurt, E. C., Grosjean, H. and Branlant, C. (1999) Pseudouridine mapping in the *Saccharomyces cerevisiae* spliceosomal U small nuclear RNAs (snRNAs) reveals that pseudouridine synthase pus1p exhibits a dual substrate specificity for U2 snRNA and tRNA. Mol. Cell. Biol. **19**, 2142–2154
11 Ganot, P., Bortolin, M. L. and Kiss, T. (1997) Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. Cell **89**, 799–809
12 Ni, J., Tien, A. L. and Fournier, M. J. (1997) Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. Cell **89**, 565–573
13 Jady, B. E. and Kiss, T. (2001) A small nucleolar guide RNA functions both in 2′-O-ribose methylation and pseudouridylation of the U5 spliceosomal RNA. EMBO J. **20**, 541–551
14 Ganot, P., Jady, B. E., Bortolin, M. L., Darzacq, X. and Kiss, T. (1999) Nucleolar factors direct the 2′-O-ribose methylation and pseudouridylation of U6 spliceosomal RNA. Mol. Cell. Biol. **19**, 6906–6917
15 Zhao, X., Li, Z.-H., Terns, R. M., Terns, M. P. and Yu, Y.-T. (2002) An H/ACA guide RNA directs U2 pseudouridylation at two different sites in the branch point recognition region in *Xenopus* oocytes. RNA **8**, 1515–1525
16 Griffey, R. H., Davis, D., Yamaizumi, Z., Nishimura, S., Bax, A., Hawkins, B. and Poulter, C. D. (1985) ^{15}N-Labeled *Escherichia coli* tRNAfMet, tRNAGlu, tRNATyr, and tRNAPhe. Double resonance and two-dimensional NMR of N^1-labeled pseudouridine. J. Biol. Chem. **260**, 9734–9741
17 Davis, D. R. (1995) Stabilization of RNA stacking by pseudouridine. Nucleic Acids Res. **23**, 5020–5026
18 Davis, D. R. and Poulter, C. D. (1991) ^{1}H-^{15}N NMR studies of *Escherichia coli* tRNA(Phe) from hisT mutants: a structural role for pseudouridine. Biochemistry **30**, 4223–4231

19 Durant, P. C. and Davis, D. R. (1999) Stabilization of the anticodon stem-loop of tRNALys,3 by an A+C base-pair and by pseudouridine. J. Mol. Biol. **285**, 115–131
20 Davis, D. R., Veltri, C. A. and Nielsen, L. (1998) An RNA model system for investigation of pseudouridine stabilization of the codon-anticodon interaction in tRNALys, tRNAHis and tRNATyr. J. Biomol. Struct. Dyn. **15**, 1121–1132
21 Tomita, K., Ueda, T. and Watanabe, K. (1999) The presence of pseudouridine in the anticodon alters the genetic code: a possible mechanism for assignment of the AAA lysine codon as asparagine in echinoderm mitochondria. Nucleic Acids Res. **27**, 1683–1689
22 Zerfass, K. and Beier, H. (1992) Pseudouridine in the anticodon G psi A of plant cytoplasmic tRNA(Tyr) is required for UAG and UAA suppression in the TMV-specific context. Nucleic Acids Res. **20**, 5911–5918
23 Perret, V., Garcia, A., Grosjean, H., Ebel, J. P., Florentz, C. and Giege, R. (1990) Relaxation of a transfer RNA specificity by removal of modified nucleotides. Nature (London) **344**, 787–789
24 Yu, Y. T., Shu, M. D. and Steitz, J. A. (1998) Modifications of U2 snRNA are required for snRNP assembly and pre-mRNA splicing. EMBO J. **17**, 5783–5795
25 Segault, V., Will, C. L., Sproat, B. S. and Luhrmann, R. (1995) *In vitro* reconstitution of mammalian U2 and U5 snRNPs active in splicing: Sm proteins are functionally interchangeable and are essential for the formation of functional U2 and U5 snRNPs. EMBO J. **14**, 4010–4021
26 Becker, H. F., Motorin, Y., Planta, R. J. and Grosjean, H. (1997) The yeast gene YNL292w encodes a pseudouridine synthase (Pus4) catalyzing the formation of psi55 in both mitochondrial and cytoplasmic tRNAs. Nucleic Acids Res. **25**, 4493–4499
27 Grosshans, H., Lecointe, F., Grosjean, H., Hurt, E. and Simos, G. (2001) Pus1p-dependent tRNA pseudouridinylation becomes essential when tRNA biogenesis is compromised in yeast. J. Biol. Chem. **276**, 46333–46339
28 Kinghorn, S. M., O'Byrne, C. P., Booth, I. R. and Stansfield, I. (2002) Physiological analysis of the role of truB in *Escherichia coli*: a role for tRNA modification in extreme temperature resistance. Microbiology **148**, 3511–3520
29 Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. Genetics **77**, 71–94
30 Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich, H. (1986) Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harb. Symp. Quant. Biol. **51**, 263–273
31 Mullis, K. B. and Faloona, F. A. (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol. **155**, 335–350
32 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
33 Lewis, J. A. and Fleming, J. T. (1995) Basic culture methods. In *Caenorhabditis elegans*: Modern Biological Analysis of an Organism. Methods in Cell Biology, vol. 48 (Epstein, H. F. and Shakes, D. C., eds.), pp. 3–29, Academic Press, New York
34 Johnstone, I. L. (1999) Molecular Biology. In *C. elegans*: a Practical Approach (Hope, I. A., ed.) pp. 201–225, Oxford University Press, Oxford
35 Barstead, R. J., Kleiman, L. and Waterston, R. H. (1991) Cloning, sequencing, and mapping of an alpha-actinin gene from the nematode *Caenorhabditis elegans*. Cell Motil. Cytoskeleton **20**, 69–78
36 Williams, B. D., Schrank, B., Huynh, C., Shownkeen, R. and Waterston, R. H. (1992) A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. Genetics **131**, 609–624
37 Bakin, A. V. and Ofengand, J. (1998) Mapping of pseudouridine residues in RNA to nucleotide resolution. Methods Mol. Biol. **77**, 297–309
38 Baumstark, T. and Ahlquist, P. (2001) The brome mosaic virus RNA3 intergenic replication enhancer folds to mimic a tRNA TpsiC-stem loop and is modified *in vivo*. RNA **7**, 1652–1670
39 Sambrook, J. and Rusell, D. W. (2001) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY
40 Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. and Green, M. R. (1984) Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**, 7035–7056
41 Hill, A. A., Hunter, C. P., Tsung, B. T., Tucker-Kellogg, G. and Brown, E. L. (2000) Genomic analysis of gene expression in *C. elegans*. Science **290**, 809–812
42 Raychaudhuri, S., Niu, L., Conrad, J., Lane, B. G. and Ofengand, J. (1999) Functional effect of deletion and mutation of the *Escherichia coli* ribosomal RNA and tRNA pseudouridine synthase RluA. J. Biol. Chem. **274**, 18880–18886
43 Huang, L., Pookanjanatavip, M., Gu, X. and Santi, D. V. (1998) A conserved aspartate of tRNA pseudouridine synthase is essential for activity and a probable nucleophilic catalyst. Biochemistry **37**, 344–351
44 Foster, P. G., Huang, L., Santi, D. V. and Stroud, R. M. (2000) The structural basis for tRNA recognition and pseudouridine formation by pseudouridine synthase I. Nat. Struct. Biol. **7**, 23–27
45 Sprinzl, M., Horn, C., Brown, M., Ioudovitch, A. and Steinberg, S. (1998) Compilation of tRNA sequences and sequences of tRNA genes. Nucleic Acids Res. **26**, 148–153
46 Lowe, T. M. and Eddy, S. R. (1997) tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. **25**, 955–964
47 Lecointe, F., Simos, G., Sauer, A., Hurt, E. C., Motorin, Y. and Grosjean, H. (1998) Characterization of yeast protein Deg1 as pseudouridine synthase (Pus3) catalyzing the formation of psi 38 and psi 39 in tRNA anticodon loop. J. Biol. Chem. **273**, 1316–1323
48 Chen, J. and Patton, J. R. (2000) Pseudouridine synthase 3 from mouse modifies the anticodon loop of tRNA. Biochemistry **39**, 12723–12730
48a Ma, X., Zhao, X. and Yu, Y.-T. (2003) Pseudouridylation (ψ) of U2 snRNA in *S. cerevisiae* is catalyzed by an RNA-independent mechanism. EMBO J. **22**, in the press
49 Guthrie, C. and Patterson, B. (1988) Spliceosomal snRNAs. Annu. Rev. Genet. **22**, 387–419
50 Kiss, A. M., Jady, B. E., Darzacq, X., Verheggen, C., Bertrand, E. and Kiss, T. (2002) A Cajal body-specific pseudouridylation guide RNA is composed of two box H/ACA snoRNA-like domains. Nucleic Acids Res. **30**, 4643–4649
51 Lafontaine, D. L. J., Bousquet-Antonelli, C., Henry, Y., Caizergues-Ferrer, M. and Tollervey, D. (1998) The box H+ACA snoRNAs carry Cbf5p, the putative rRNA pseudouridine synthase. Genes Dev. **12**, 527–537
52 Heiss, N. S., Knight, S. W., Vulliamy, T. J., Klauck, S. M., Wiemann, S., Mason, P. J., Poustka, A. and Dokal, I. (1998) X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. Nat. Genet. **19**, 32–38
53 Gu, J., Patton, J. R., Shimba, S. and Reddy, R. (1996) Localization of modified nucleotides in *Schizosaccharomyces pombe* spliceosomal small nuclear RNAs: modified nucleotides are clustered in functionally important regions. RNA **2**, 909–918
54 Reddy, R. and Busch, H. (1988) Small nuclear RNAs: RNA sequences, structure, and modifications. In Structure and Function of Major and Minor snRNPs (Birnstiel, M., ed.), pp. 1–37, Springer Verlag, Berlin
55 Koonin, E. V. (1996) Pseudouridine synthases: four families of enzymes containing a putative uridine-binding motif also conserved in dUTPases and dCTP deaminases. Nucleic Acids Res. **24**, 2411–2415

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