A novel unanticipated type of pseudouridine synthase with homologs in bacteria, archaea, and eukarya

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ABSTRACT

Putative pseudouridine synthase genes are members of a class consisting of four subgroups that possess characteristic amino acid sequence motifs. These genes have been found in all organisms sequenced to date. In *Escherichia coli*, 10 such genes have been identified, and the 10 synthase gene products have been shown to function in making all of the pseudouridines found in tRNA and ribosomal RNA except for tRNA^{Glu} pseudouridine13. In this work, a protein able to make this pseudouridine was purified by standard biochemical procedures. Amino-terminal sequencing of the isolated protein identified the synthase as YgbO. Deletion of the *ygbO* gene caused the loss of tRNA^{Glu} pseudouridine13 and plasmid-borne restoration of the structural gene restored pseudouridine13. Reaction of the overexpressed gene product, renamed TruD, with a tRNA^{Glu} transcript made in vitro also yielded only pseudouridine13. A search of the database detected 58 homologs of TruD spanning all three phylogenetic domains, including ancient organisms. Thus, we have identified a new wide-spread class of pseudouridine synthase with no sequence homology to the previously known four subgroups. The only completely conserved sequence motif in all 59 organisms that contained aspartate was GXKD, in motif II. This aspartate was essential for in vitro activity.

Keywords: Glutamate tRNA; pseudouridine13; TruD; *Escherichia coli*

INTRODUCTION

Pseudouridine (5-ribosyluracil; Ψ), is the most common modified nucleoside found in RNA (Rozenski et al. 1999). It is made by isomerization of selected uridines in a preformed polynucleotide by a set of enzymes called Ψ synthases. No energy input is required for the reaction, and no cofactors are involved, except that zinc is essential for a yeast synthase (Arluison et al. 1998) and in eukaryotes, Ψ formation in ribosomal RNA requires the participation of at least three auxiliary proteins and a guide RNA (Kiss 2001). The enzymes are specific for particular uridines in an RNA and also for the RNA species. Commonly, each Ψ in an RNA is made by its own synthase, but there are a number of cases known in which a single synthase makes more than one Ψ (Kammen et al. 1988; Conrad et al. 1998; Huang et al. 1998a; Lecointe et al. 1998; Motorin et al. 1998; Raychaudhuri et al. 1998; Chen and Patton 1999, 2000; Hellmuth et al. 2000). The reverse, more than one synthase

making the same Ψ , has so far not been found. Typically, Ψ synthases are specific for a single class of RNA, but rare exceptions to this rule also exist (Wrzesinski et al. 1995b; Massenet et al. 1999).

As a result of this high specificity of Ψ synthases and the frequent occurrence of Ψ in tRNA, rRNA, and sn(o)RNA, multiple Ψ synthases can be found in most cell types. For example, prior to this work, *Escherichia coli* was known to have 10 experimentally proven Ψ synthases (Del Campo et al. 2001). The maximum number so far found in bacteria is 12, in *Vibrio cholerae* (Ofengand 2002) and in *Xanthomonas* species (J. Ofengand, unpubl.). The synthases in the above two organisms were not directly identified, but instead, putative synthase genes were identified by amino acid sequence homology with experimentally identified synthases (Gustaffson et al. 1996; Koonin 1996; Ofengand and Rudd 2000; Ofengand 2002). This technique is quite powerful, for example, 6 of the 10 *E. coli* synthases were predicted by this method and subsequently confirmed experimentally. By this approach, Ψ synthase genes have been identified in all of the completely sequenced genomes from Bacteria, Archaea, and Eukarya that are publicly available (Ofengand 2002; J. Ofengand, unpubl.). Thus, all forms of life appear to have Ψ synthases, and by extension should have Ψ residues. This in turn implies a functional role for Ψ that is both ancient and that crosses phyla boundaries.

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The number of Ψ synthases in a given organism identified by this approach is quite variable. As noted above, *V. cholerae* and *Xanthomonas sp.* have 12 putative synthases. On the other hand, the Crenarchaeota so far sequenced, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Sulfolobus solfataricus*, and *Sulfolobus tokodaii* have but a single synthase gene that is highly homologous to that for yeast Cbf5 and its equivalent in other eukaryotes. This is the Ψ synthase believed to function in conjunction with guide RNAs to make Ψ in ribosomal RNA of eukaryotes. This result, on the one hand, implies that guide RNAs are used in these archaeal organisms, but, on the other hand, raises the question of where is (are) the synthase(s) for making Ψ in their tRNA. Although it is not known experimentally whether Ψ is present in the tRNA of these organisms, it seems highly unlikely that Ψ would be completely absent. One possibility is that there are Ψ synthases remaining to be discovered that are not detectable by the homology searches used up to now because their amino acid sequences are truly distinct. A hint that such a possibility could occur comes from the X-ray structures of TruA (Foster et al. 2000), TruB (Hoang and Ferré-D'Amaré 2001), and RsuA (Sivaraman et al. 2002), which show considerable structural similarity including the position of the putative catalytic aspartate residue, despite the fact that the sequence of TruA is sufficiently different from the others so as to place TruA in a family separate from the TruB, RsuA, and RluA families (Ofengand and Rudd 2000), although it is still recognizable as a Ψ synthase.

In *E. coli*, although the function of all 10 Ψ synthases has now been assigned, no synthase for Ψ 13 of tRNA^{Glu} has yet been identified (Del Campo et al. 2001). Moreover, individual deletion of each of the 10 synthase genes failed to cause the loss of this Ψ from tRNA^{Glu} (Y. Kaya, data not shown). We hypothesized that this synthase might have an amino acid sequence that is completely different from the Ψ synthases known up to now and searched for it by the standard biochemical procedures we used previously to identify three other *E. coli* Ψ synthases (Nurse et al. 1995; Wrzesinski et al., 1995a,b).

In this study, we identify the gene encoding the synthase that generates Ψ 13 of tRNA^{Glu}. We show that although this protein has no amino acid sequence similarity to known Ψ synthases, it is homologous to a new class of 58 proteins of previously unknown function whose genes are found in organisms from all phyla. Thus, this work defines at least one potential function for these proteins. Furthermore, as none of the known RNA-binding motifs can be detected among the six conserved motifs of this class of proteins, they should contain novel RNA-binding sequences. These sequences may identify other classes of RNA-binding proteins including, but not limited to, other RNA base-modifying enzymes. We also identify an essential aspartate residue found in a short sequence motif common to all members of this new class, but which is completely different from the essential aspartate-containing motif II of the

known Ψ synthases (Huang et al. 1998b; Ofengand et al. 2001b; Ofengand 2002). This new sequence motif is likely to be part of an alternative structural design for the catalytic center of Ψ synthases. These observations expand our knowledge of the variety of Ψ synthases, and may explain the apparent paucity of these enzymes in certain organisms.

RESULTS

Purification of the Ψ 13-forming enzyme and identification of the gene

Isolation of the Ψ 13-forming activity from *E. coli* was done by classical biochemical means. The only convenient quantitative assay for Ψ formation is the release of ³H from position 5 of uridine in the process of isomerization to Ψ (Cortese et al. 1974). Because in *E. coli*, tRNA^{Glu} is the only tRNA with a Ψ 13, 5-[³H]uridine-labeled transcripts of this gene were used to assay for activity. Moreover, as tRNA^{Glu} has two Ψ , at positions 13 and 55, the source of the cell-free extract was the Δ truB strain, which does not make Ψ 55 (Gutgsell et al. 2000). Therefore, aside from ³H release due to other activities such as m⁵U formation, which requires S-adenosyl methionine, not included in the assay, ³H release activity should be indicative of Ψ 13 formation. A purification procedure previously used successfully for other *E. coli* Ψ synthases (Nurse et al. 1995; Wrzesinski et al. 1995a,b) was adopted. The final step of the purification is shown in Figure 1. A single peak of activity was obtained that correlated well with a major protein band at ~40 kD. This is only slightly larger than that found for other Ψ synthases (Del Campo et al. 2001). Amino-terminal sequencing of this band yielded the sequence MIEFDNLTYL, an exact match only to the predicted product of the *E. coli* ygbO gene. The calculated MW, 39.1 kD, is in good agreement with that for the protein band on SDS gels (Fig. 1B). Proof that the ygbO gene product is solely responsible for Ψ 13 formation was obtained by deletion of the gene.

Ψ sequencing analysis of a ygbO deletion strain

The ygbO gene was deleted by the method of Datsenko and Wanner (2000). Deletion was verified by kanamycin resistance, and the increased size of the PCR product produced by amplification from the amino- and carboxy-terminal ends of the gene (data not shown). Deletion had no obvious effect on growth. To be sure that the deletion strain as isolated was not a pseudorevertant with normal growth properties, it was backcrossed to wild type by P1 transduction. Of 88 kanamycin-resistant colonies obtained, none showed a smaller colony phenotype. Therefore, if a second-site mutation to produce a wild-type colony phenotype existed, it must cotransduce at a frequency >98.9%, which means it must be within 350 bp of the truD gene (Bachman et al. 1976). Analysis of tRNA^{Glu} from this deletion strain

FIGURE 1. MonoS column purification of tRNA^{Glu} Ψ13 synthase. (A) Activity (□) was tested as described in Materials and Methods and units were expressed as described. Protein was measured as A₂₈₀ (■). (B) Equal volumes of the column fractions indicated were electrophoresed in SDS gels. (St) MW standards with values as indicated. (Arrow) protein band taken for sequencing.

and the wild-type control showed that Ψ13, present in the wild type, was absent from the deletion strain (Fig. 2). Both strains were transformed with pTrc99A as controls for the D80D pair of lanes in which pTrc99A carrying only the wild-type structural gene was used to transform the deletion strain. The *ygbO* structural gene restored Ψ13 formation (Fig. 2), showing that the *ygbO* product is necessary and sufficient for Ψ13 formation. Therefore, we have renamed YgbO as TruD, the fourth tRNA-specific Ψ synthase.

In vitro activity of overexpressed and affinity purified TruD

To test the in vitro activity of TruD, the gene was subcloned into pET28a and overexpressed in BL23(DE3) cells. Good expression was obtained with ~70% in the soluble phase. Because this construct places a His tag at the amino terminus of TruD, it was possible to purify it by affinity chromatography on a Ni-binding resin. The in vitro substrate used was a 5-[³H]uridine-labeled in vitro transcript of tRNA^{Glu}, so that Ψ formation could be followed by ³H release. As shown in Figure 3A, reaction occurred readily and reached a stable plateau at ~1 mole tritium released per mole of transcript added, exactly as expected. Addition of

more enzyme at 120 min did not change the amount of reaction, showing that the kinetic plateau was not due to enzyme inactivation. Two mutant proteins in which D80 had been mutated to N or T, which were overexpressed and purified in exactly the same way, were completely inactive, even when present in a 10-fold excess over that of the wild type. This result will be discussed below. RNA incubated with wild-type or mutant TruD for 200 min (Fig. 3A), and RNA incubated for the same time without TruD were sequenced for Ψ. Figure 3B shows that the product of the in vitro reaction is Ψ13, just as it is in vivo.

Effect of the absence of TruD and Ψ13 on the cell

Deletion of *truD* causes the loss both of the gene product, TruD, and the synthase product, Ψ13. What is the physiological effect of this loss? One excellent parameter for measuring effects on cells is their growth rate when they are dividing at the maximum rate allowed by the growth conditions, that is, in exponential phase. Therefore, growth rates of the wild-type and deletion strains were determined. Cells were grown with aeration in LB or M9/glucose/Mg medium (Gutgsell et al. 2000) at 25, 37, and 42°C, and

FIGURE 2. Ψ sequence analysis of the *truD* deletion strain (Δ*truD*). Deletion of the *ygbO* (renamed *truD*) gene in MG1655, construction of wild-type-containing pTrc99A, transformation, and growth of the transformed cells, isolation of RNA, and Ψ sequencing were all performed as described in Materials and Methods. The primer was complementary to tRNA^{Glu} residues 17–33. The Δ*truD* strain was transformed with pTrc99A with no insert (0) and with the wild-type *truD* structural gene (D80D). As a control, wild-type MG1655 was transformed with pTrc99A with no insert (0). Cells at an A₆₀₀ of 0.6 were induced with 2 mM IPTG for 3 h at 37°C before phenol extraction of the RNA. Reaction with (+) or without (–) CMC followed the standard sequencing protocol. (A, G, C, U) RNA sequencing lanes. The arrow shows the position of the stop one residue 3' to Ψ13.

FIGURE 3. In vitro activity of the wild-type (D80D) and two mutant TruD synthases (D80N, D80T). (A) ^3H release activity of wild-type and mutant TruD. A 5- ^3H uracil-containing tRNA^{Glu} transcript was prepared and pseudouridine formation measured as release of ^3H as described in Materials and Methods. Substrate concentration was 100 nM in a reaction volume of 0.1 mL. Wild-type and mutant recombinant synthases were overexpressed from pET28a plasmids and purified (see Materials and Methods). A total of 0.05 μg of D80D (◆), no enzyme (◇), and 0.54 μg of D80N and D80T (triangles and circles, respectively) were added. A total of 0.15 μg of wild-type or mutant enzyme was added to the reaction at 120 min (arrow). (B) Ψ sequence analysis of the site of in vitro Ψ formation on tRNA^{Glu}. RNAs that had been reacted with the recombinant wild-type and mutant enzyme to completion (200 min) were obtained by phenol extraction of the mixture and analyzed by the sequencing technique described in Materials and Methods. RNA was reacted with (+) or without (−) CMC following the standard sequencing protocol. (A, G, C, U) RNA sequencing lanes. The arrow shows the position of the stop one residue 3' to Ψ 13.

assayed spectrophotometrically at 600 nm over 3–6 doublings. No difference in growth rate between the two strains was found under any of the conditions. Moreover, growth competition studies, done as described previously (Gutgsell et al. 2000), in LB at 37 or 42°C did not show any effect of the deletion (data not shown).

Homologs of TruD are found in other genomes

The online version of PSI-BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) at the National Center for Biotechnology Information (NCBI) was used to detect homologs of TruD. Initially, the amino acid sequence of TruD was searched against the nonredundant protein database at the NCBI site, and iterated to convergence with the default threshold (0.005). This initial search identified 68 sequences with an E-value better than the threshold ranging from e^{-132} to e^{-8} , and 16 sequences with E-values above the threshold ranging from 0.027 to 9.3. To expand the number of homologs, the sequences with E-values greater than the default threshold value were individually searched against the database with PSI-BLAST. None of these sequences were able to identify more than a few members of the 68-member list. Therefore, these sequences were excluded from the class.

Subsequent analysis of the class was done by multiple alignment using the Clustal X version 1.81 software program (Jeanmougin et al. 1998). Initially, a phylogenetic tree was created, and using the tree, all members were multiple aligned to each other. Final adjustment of the alignment was made manually. Even though the PSI-BLAST search found statistically significant homology among the 68 members, six of the sequences (gi|20343688|, gi|9506681|, gi|14250243|, gi|20823027|, gi|9366673|, and gi|20957799|) did not align at the amino terminus. Therefore, these members were excluded in the final alignment. Although it is possible that these proteins were incomplete in the database, we did not attempt to identify the missing sequence in the corresponding genome to verify this. Additionally, three pairs of sequences were found to be identical or nearly so. Two proteins (gi|16554460| and gi|10579883|) from *Halobacterium* sp. NRC-1 are the products of the same gene, VNG0243C, but one (gi|10579883|) is 141 amino acids longer at the amino terminus. Two proteins (gi|16130652| and gi|15803262|) from *E. coli* (strain K-12 and EDL933, respectively) differed in two amino acids

at residues 176 (Gln versus Leu) and 242 (Ala versus Val), respectively. Two proteins (gi|16761700| and gi|16766234|) were from different subgroups of *Salmonella* (*enterica* serovar Typhi and *typhimurium* LT2, respectively) and differed only at amino acid residue 61 (Val versus Ala, respectively). One representative of each pair was used in the final alignment of 59 sequences (Fig. 4).

There are 24 TruD homologs in Eubacteria, 19 in Archaea, and 16 are in eukaryotes. All are from complete genome sequences except for the archaeal *Ferroplasma acidarmanus* and seven from eukaryotes that are from incomplete genome sequences. Even early diverging organisms such as *Aquifex aeolicus* of the Eubacteria, *Encephalitozoon cuniculi* of the Eukarya, and all of the sequenced Archaea have a TruD homolog, suggesting that like the canonical Ψ synthases, this new class has ancient origins. Although all of the analyzed Archaea have a TruD homolog, as do all of the sequenced eukaryotes except *Guillardia theta*, homologs in the Eubacteria were found mainly in the γ and δ - ϵ subdivisions of the Proteobacteria. However, homologs were found in *A. aeolicus* and *Deinococcus radiodurans*, two organisms among the earliest to branch off in the bacterial kingdom. Therefore, the absence of TruD homologs from major branches of the Eubacteria sug-

gests that they were evolutionarily lost rather than never present.

Six motifs were detected, as shown in Figure 4. Beside these motifs, regions showing some homology were found in amino-terminal neighboring residues of motif II, between motifs IV and V, and between motifs V and VI. Motif I is missing in five organisms, motif V in one, and motif VI is absent in two. Of the 10 completely conserved amino acids, 4 are found in motif II, and 3 are in the sequence, GXKD, which also contains the only completely conserved aspartate. Analysis of this motif is discussed below.

Aspartate 80 in the GXKD motif of TruD is essential for Ψ -forming activity

The multiple sequence alignment of Figure 4 identified 10 completely conserved amino acids, 3 of which are in the motif GXKD. Because aspartate is an essential residue in all other known Ψ synthases (Ofengand and Rudd 2000; Ofengand et al. 2001b), and may be the catalytic residue (Gu et al. 1999; Hoang and Ferré-D'Amaré 2001), we postulated that the aspartate in the GXKD motif, D80, which is the only completely conserved aspartate in all of the motifs, might be essential for activity. This was tested in vitro by constructing the appropriate mutant gene in pET28a and overexpressing the protein with an amino-terminal His tag for affinity purification in the same way as for the wild type. Figure 3 shows that in vitro, both D80T and D80N mutant TruD were completely inactive, even when a large excess of protein was added. Clearly, D80 is essential for Ψ activity. We suggest that D80 may be the catalytic aspartate in TruD, and that when the three-dimensional structure of this protein is determined, D80 will be found at the equivalent location in space and with a similar protein fold as the catalytic aspartate and surrounding protein fold in the other three known structures (Mueller 2002).

DISCUSSION

Specificity of the *E. coli* tRNA Ψ 13 synthase

The characterization of this synthase accounts for the last known site for Ψ in *E. coli* rRNA or tRNA. The 10 previously identified synthases accounted for all of the other sites (Del Campo et al. 2001). In vivo, it is the only synthase capable of making this Ψ , as deletion of the *truD* gene caused Ψ 13 to disappear and restoration of the gene on a plasmid caused it to reappear. Likewise, TruD must be unable to make Ψ at any of the other known in vivo sites in tRNA or rRNA, as deletion of the appropriate synthase gene causes loss of its Ψ while in the continued presence of *truD*. However, whether any of the various small RNAs in *E. coli* (Argaman et al. 2001; Rivas et al. 2001; Wassarman et al. 2001) contain Ψ , and whether any are made by this synthase, is still an open question.

Function of Ψ 13 in tRNA

No growth effect was detected upon deletion of *truD*, either in exponential growth rate at temperatures from 25 to -42°C in rich or minimal medium, or in competition with wild type in rich medium at 37 or 42°C . Nevertheless, considering the wide distribution of Ψ 13 in tRNA (see below), its location at the end of a base-paired stem connected by a loop, and the known propensity of Ψ for structural stabilization (Meroueh et al. 2000; Newby and Greenbaum 2002, and references therein), it might be that certain tRNAs, like *E. coli* tRNA^{Glu}, require this particular stabilization. Analysis of the database of sequenced tRNAs (Sprinzl and Vassilenko 2002) reveals a suggestive relationship between the distribution of U or Ψ at position 13 and the nature of the putatively base-paired residue at position 22. Excluding only animal mitochondrial tRNAs, when U22 is present, Ψ is found in 45 of 47 (96%) of the cases. With G22, Ψ is present in 32 of 45 (71%) of the tRNAs, and with A22, Ψ is only found in 8 of 32 (25%) examples. Thus, Ψ appears needed when the opposite partner is U, is somewhat preferred when making the G-U wobble pair, and is actually not preferred for making an A-U base pair. This may explain why the lack of Ψ 13 in *E. coli*, which has Ψ 13 in only one tRNA, tRNA^{Glu}, and even that one is opposite G22, has no detectable growth effect. In this case, it may be that the effect only becomes manifest under special conditions, when tRNA^{Glu} might become limiting for some metabolic process. In other organisms, like *Saccharomyces cerevisiae*, which has five tRNAs with a Ψ 13:U22 base pair, deletion of the Ψ 13 synthase gene might have a detectable growth defect even under laboratory growth conditions.

Prevalence of Ψ 13 and Ψ 13 synthases

Although only tRNA^{Glu} contains Ψ 13 in *E. coli*, Ψ 13 is actually widely distributed both among tRNAs and species. tRNAs for 16 of the 21 (including Met_i) amino acids have Ψ 13, and it is found in tRNAs from all three major phyla of life, the Eubacteria, Archaea, and Eukarya (Sprinzl and Vassilenko 2002). Presumably, all of these organisms have a Ψ synthase for making Ψ 13. In fact, a partially purified enzyme specific for Ψ 13 in *S. cerevisiae* has been described (Samuelsson and Olsson 1990). However, there is no information available on whether a conventional Ψ synthase, that is, a member of the TruA, TruB, RsuA, or RluA families (Ofengand and Rudd 2000; Ofengand et al. 2001b), or one homologous to TruD is used. There is one homolog of TruD in *S. cerevisiae*, gi6324817 (YOR243c), see Figure 4, which could be the Ψ 13 synthase. However, that gene was shown recently to make a synthase responsible for Ψ 35 in yeast U2 snRNA (Ma et al. 2003). Thus, unless this synthase is a dual specificity protein like *E. coli* RluA (Wrzesinski et al. 1995b) or yeast Pus1p (Massenet et al. 1999), there must still be another sequence class of Ψ synthase in yeast that is responsible for tRNA Ψ 13.

FIGURE 4. (Legend on facing page)

FIGURE 1

A new class of Ψ synthase

Identification of *ygbO* as *truD*, the gene for a Ψ synthase with no amino acid sequence homology to the four known classes, TruA, TruB, RsuA, and RluA, provides a potential solution to the problem of too few Ψ synthases in the archaea. Analysis of 16 archaeal genomes for conventional Ψ synthases (Ofengand 2002; J. Ofengand, unpubl.) has shown that the 4 crenarchaeota have but one synthase, which is of the yeast Cbf5 type. This type of synthase works normally with guide RNAs to make Ψ in rRNA (Kiss 2001) and is not known to recognize tRNA. The 12 euryarchaeota have, in addition to the Cbf5-like synthase, a single TruA class enzyme. This class makes Ψ in the tRNA anticodon at residues 38–40 in *E. coli* (Kammen et al. 1988), yeast (Leconte et al. 1998), and mouse (Chen and Patton 2000), but is not known to make any other tRNA Ψ . However, known archaeal tRNAs have Ψ at eight positions, needing at least four synthases by conventional specificity criteria. Moreover, Ψ 55 occurs in *Methanothermobacter thermoautotrophicus*, *Thermoplasma acidophilum*, *Pyrococcus furiosus*, *Halobacterium cutirubrum*, *Haloferax volcanii*, and *Halococcus morrhua* (references cited in Gutgsell et al. 2000), yet the former three lack a recognizable TruB class synthase. This may be true for the latter three as well, but the genome sequencing data is not available. Eighteen archaeal genomes have homologs of TruD (Fig. 4), including *M. thermoautotrophicus*, *T. acidophilum*, and *P. furiosus*, suggesting that some may be responsible for Ψ 55 synthesis rather than for Ψ 13. Similarly, three eubacteria, *Helicobacter pylori*, *Mycoplasma genitalium*, and *Mycoplasma pneumoniae*, and one eukaryote, *Caenorhabditis elegans*, lack a putative TruB synthase for making Ψ 55 (Ofengand 2002). Two of the four, *H. pylori* and *C. elegans*, have TruD homologs (Fig. 4). On the other hand, *H. volcanii*, *H. cutirubrum*, *M. thermoautotrophicus*, *T. acidophilum*, and *H. morrhua* are known to have tRNAs with Ψ 13, so that it is also possible that the TruD homologs found in archaea make Ψ 13. In that event, Ψ 55 might be made by a synthase of a still different amino acid sequence class. As mentioned above, the yeast Ψ 13 synthase might be a member of such an additional class also, as there was only one TruD homolog in yeast, and that was the synthase for U2 snRNA Ψ 35. It is also worth noting that

whereas *E. coli* has TruD, it has no other genes homologous to it.

It is still too soon to make firm conclusions about the number of Ψ synthase classes and how they are related. However, the discovery of TruD makes it clear that additional classes could exist. Thus, the current work vastly expands the potential for discovering still more classes of pseudouridine synthases. This discovery also reinforces the fact that, whereas bioinformatic analysis is a very powerful tool for discovery of the function of genes, traditional biochemistry still has an important role to play.

Functional motifs

The only motif with a conserved aspartate residue is motif II (Fig. 4). The lack of in vitro activity of TruD with this aspartate mutated to asparagine or threonine (Fig. 3) suggests that this motif may be at the catalytic center of this new family of Ψ synthases, as is aspartate in a different sequence context in the conventional Ψ synthases. Alternatively, however, the mutations could have blocked the binding of the mutant synthases to their substrate tRNA rather than affecting the catalytic center. Further studies are needed.

To detect any known RNA-binding motifs, TruD was searched against the Fold Library SCOP version 1.53 (Kelley et al. 2000) at the 3D-PSSM server (<http://www.sbg.bio.ic.ac.uk/~3dpssm/>) and with the three-dimensional model-building program 3D-JIGSAW (Contreras-Moreira and Bates 2002), (<http://www.bmm.icnet.uk/~3djigsaw/>). These searches did not identify any structural motifs at E values <0.05. The Conserved Orthologous Group (COG) database (<http://ncbi.nlm.nih.gov/COG>) was also searched using TruD as the query. A subgroup, COG0585, was identified, which contained TruD and 21 other proteins. All were members of the group in Figure 4. However, no experimental data exists for the members (except for TruD) nor do they exhibit any predicted features. Additionally, a PSI-BLAST search that reached convergence at iteration 4 failed to detect any proteins with the predicted RNA-binding domains S4, PUA, THUMP, or TRAM. These domains are found in various 4-thiouridine and pseudouridine synthases

FIGURE 4. Sequence alignment of TruD homologs from other genomes. The alignment was constructed as described in the text. The identity of each homolog is given by the *gi* number followed by the organism code taken from the Swiss-Prot site (<http://www.expasy.org/cgi-bin/speclist>), except that MICDE is *Microbulbifer degradans* 2-40; MAGS1 is *Magnetococcus* sp. MC-1; PLAYY is *Plasmodium yoelii* yoelii. There are six motifs numbered from the amino terminus to the carboxyl terminus as I to VI. The numbers between motifs II and VI are the number of amino acid residues between each motif. The numbers to the left of motif I are the number of residues to the amino terminus. Those to the right of motif VI are the number to the carboxyl terminus. Amino acids conserved 100% are white on black, amino acids with a >80% consensus are black on gray. If there is no single amino acid residue with a >80% consensus, amino acids are classified as polar (p: KRHDEQNST), hydrophobic (h: ALICVMYFW) or aliphatic as a subset of hydrophobic (a: ALICVM) as shown in the CONSENSUS line. If any column is not p, h, or a with a >80% consensus, then the column is classified according to side-chain size as tiny as a subset of small (t: GAS), small (s: GASCDNPSTV), or big (b: QERKYMFWLI). Residues that fit into a column classification are shown in black. Those that do not fit into the classification of its corresponding column are shown in gray. The secondary structure at the top of the alignment is the output of the secondary structure prediction program PHD (Rost and Sander 1993, 1994) using TruD as the query with (e) β -strand, (c) coil, and (h) helix. The most confident structure predictions (>82% accuracy) are shown in uppercase letters. The consensus sequence and a summary of the motifs are shown below the alignment.

and RNA methylases (Aravind and Koonin 1999, 2001; Anantharaman et al. 2001). Likewise, none were found with KH (Siomi et al. 1993) or RRM (Birney et al. 1993) RNA-binding motifs. Rather, all members of the TruD family made a closed set of a new protein group with no function predicted or identified for any one of its members. No members of the PsuX or PsuY families, two groups of proteins distantly related to the TruB family (Watanabe and Gray 2000), were found in the TruD set of Figure 4 either. This suggests that this new family should have a different type of RNA-binding motif than described heretofore.

On the other hand, a common feature of many RNA-base-modifying enzymes is the fusion of specific RNA-binding domains to the respective catalytic domains (Aravind and Koonin 1999). For example, many thiouridine and pseudouridine synthases and RNA methylases share a common domain that is predicted to be an RNA-binding domain (Aravind and Koonin 1999, 2001; Anantharaman et al. 2001). Binding and catalytic domains can be two separate functions in an RNA base-modifying protein molecule. One would identify the target RNA and the other would modify the base to a specific form, but both of the domains would be required for RNA base-modifying enzymes to function properly. Thus, it is possible that the RNA-binding domains of the members of the TruD family are sufficiently different from each other that alignment at their RNA-binding domains fails.

MATERIALS AND METHODS

Strains

MG1655 was the wild-type *E. coli* strain. MG1655 Δ truB::kan (Gutgsell et al. 2000) was used to make the cell extract for purification of the Ψ 13 synthase. NovaBlue (Novagen) was the host in all initial transformation experiments. BL21(DE3) (Novagen) was used for overexpression of recombinant TruD protein.

Materials

5-[³H]UTP (15.3 Ci/mmol) was from Amersham. γ -[³²P]ATP (3000 Ci/mmol) was from Perkin Elmer. Restriction enzymes were from New England Biolabs. Other reagents were obtained as noted.

Buffers

Buffer A is 10 mM Hepes (pH 8.0), 10 mM MgCl₂, 5 mM mercaptoethanol, and 0.1 mM EDTA. Buffer B is 20 mM Hepes (pH 8.0), 20 mM NH₄Cl, 5 mM mercaptoethanol, 0.1 mM EDTA, and 10% glycerol. Buffer C is buffer B, but at pH 7.8. Buffer LB is 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromophenol blue.

Pseudouridine formation assay

Reactions contained 50 mM HEPES (pH 7.5), 100 mM NH₄Cl, 5 mM Mg(OAc)₂, 100 nM 5-[³H]uracil-containing tRNA^{Glu} pre-

pared by in vitro transcription, 5 mM DTT, 400 units/mL RNasin (Promega), and enzyme or extract. Incubation was at 37°C. Reactions were stopped by addition of 100 μ L aliquots to 1.0 mL of 12% acid-washed Norit A (Sigma, cat. no. C-4386) in 0.1 N HCl. Samples were mixed and allowed to stand at room temperature for 5 min and then centrifuged to remove the charcoal, and the supernatant was passed through a PTFE filter assembly (0.2 μ m, cat. no. 187 1320, Nalge Nunc International). The charcoal pellet was washed two times with 1.0 and 0.5 mL of 0.1 N HCl, each time passing the supernatant wash through the same filter assembly. The washes and supernatant were combined and counted. The value at 0 time without enzyme was subtracted from all samples. One unit of activity is that amount of enzyme catalyzing the release of 1 pmole of ³H to the supernatant in 30 min at 37°C.

Plasmids

The *truD* gene was PCR amplified from MG1655 genomic DNA using pfu turbo polymerase (Stratagene) and cloned into the *Nco*I and *Bam*HI sites of pTrc99A (Pharmacia) and the *Nde*I and *Bam*HI sites of pET28a (Novagen). The carboxy-terminal primer (5'-CAGGATCCCTTTCGCGCAGCGTTTGTATACCG-3') was the same for insertion of the gene in both plasmids, and incorporated a *Bam*HI site (italics) followed by the complement of residues 43–21 (numbering begins after the TAA stop codon). The amino-terminal primer for cloning into pTrc99A was 5'-CTCGGTCTC GCATGATTGAGTTTGATAATCTCACTTACCTC-3', incorporating a *Bsa*I site (italics) two nucleotides before the AUG start codon (underlined). The amino-terminal primer for cloning into pET28a was 5'-CCACTGGCCATATGATTGAGTTTGATAATCTCACTTACCT-3', incorporating an *Nde*I site (italics) spanning the AUG start codon (underlined).

D80T and D80N mutants of *truD* were made in pET28a using the Quick-change XL site-directed mutagenesis kit (Stratagene) according to the instruction manual provided. For *truD*(D80T) and *truD*(D80N), the mutagenic primers were 5'-CAGCTTCGC TGGGCAAAAAACCAACATGCTGTTACGG-3', and 5'-CAG CTTCGCTGGGCAAAAAACCAACATGCTGTTACGG-3', respectively. Each mutagenic primer incorporated base changes (underlined in the primer sequence) necessary to mutate the desired aspartate residue. All of the mutant plasmids were verified by DNA sequencing.

pUC7a and pUC7b vectors were created by cloning a 41-nucleotide long dsDNA fragment into the *Sma*I site of pUC19 (New England Biolabs, Inc.). The dsDNA fragment (5'-pTAATACGACTCACTATAGGA¹ATGCTTCAGGGATCTGC AC-3') contained a T7 promoter (underlined) and recognition sites (italics) for *Bbs*I and *Bsg*I restriction enzymes, respectively. The top strand (shown) and the complementary strand were obtained from Invitrogen with 5' phosphate ends. These oligomers were annealed to each other in a 1:1 molar ratio prior to blunt-end cloning into pUC19 at the *Sma*I site. The direction of cloning was determined by DNA sequencing. When the insert as written was in the same direction as pUC19 numbering, it was named pUC7a. Inserts in the opposite direction were named pUC7b. The plasmids were then cleaved (arrow) with *Bsg*I. The resulting 2-nucleotide 3' overhang (shown in bold) was removed with DNA polymerase I Klenow fragment (New England Biolabs, Inc.) and dephosphorylated with shrimp alkaline phosphatase (Roche Applied Sciences). These linearized plasmids can be used as blunt-end

acceptor vectors to clone any desired DNA fragment for transcription by T7 polymerase with two extra G residues at the 5' end using cleavage at the *Bbs*I site for runoff transcription. Plasmid pUCt7a was used for tRNA^{Glu} transcription. The gene was PCR amplified from MG1655 genomic DNA using pfu turbo polymerase. The 5' primer (5'-pTCCCTTCGTCTAGAGGCC-3') was the sequence of residues 2–21 and contained a 5' phosphate. The 3' primer (5'-pTGGCGTCCCCTAGGGGATTC-3') was the complement of residues 76–57 and also contained a 5' phosphate. The direction of cloning was tested by DNA sequencing. The plasmid containing the insert in the correct direction was named pUCt7a::tRNA^{Glu}.

RNA transcripts

The tRNA^{Glu} transcript was prepared by linearization of pUCt7a::tRNA^{Glu} with restriction enzyme *Bbs*I, and transcribed using the T7-MEGAshortscript kit (Ambion). The reaction contained 7.5 mM each of ATP, CTP, UTP, and GTP, 200 nM of plasmid, 800 μ Ci/mL 5-[³H]UTP, 2 μ L T7-MEGAshortscript enzyme mix and 2 μ L of the 10 $\times$ transcription buffer from the Ambion kit in a final volume of 20 μ L. Incubation was at 37°C for 10–12 h. The template plasmid was removed by addition of RNase-free DNase to a concentration of 100 units/mL at 37°C for 20 min. RNA transcripts were then purified by phenol extraction, ethanol precipitation, and G-50 gel filtration.

Purification of the Ψ 13 synthase

Forty grams of *E. coli* MG1655:: Δ *truB* frozen cell pellet, harvested in midlog phase, was thawed at 4°C with 30 mL of buffer A. After removal of liquid by centrifugation, the cells were suspended in 80 mL of buffer A, 10% glycerol, 0.67 mM PMSF (Sigma), plus eight tablets of protease inhibitor (Complete, Mini from Roche Applied Science), and passed through a French press at 18,000 psi. (1240 bar). Ribosomes and cell debris were removed by centrifugation at 45,000 rpm for 2 h in a Ti60 rotor at 4°C. To the supernatant (S200) was added one-seventh volume of 20% streptomycin sulfate adjusted to pH 7.5, and the mixture stirred at 4°C for 30 min. The streptomycin supernatant (78 mL) was recovered by centrifugation and precipitated with solid ammonium sulfate (43.6 g). The mixture was adjusted to pH 7.5 with NH₄OH and stirred overnight at 4°C. The precipitate was collected by centrifugation, dissolved in 20 mL of buffer B, and dialyzed against the same buffer to remove ammonium sulfate.

The dialyzed sample was loaded at 0.5 mL/min on a 1.5 $\times$ 45-cm column of DEAE Sepharose CL6B (Pharmacia) equilibrated with buffer C and eluted with a linear gradient of 1 mM/mL NH₄Cl in buffer C. Synthase activity eluted in one major peak at 250 mM NH₄Cl. Peak fractions were pooled, changed, and concentrated by dialysis against buffer C plus 10% polyethylene glycol 8000 (Sigma). The activity recovered was 80% of the column input. The sample (10 mL) was then loaded on an 8-mL MonoS (Pharmacia) column equilibrated in buffer C and eluted at 1 mL/min with a linear gradient of 3.33 mM NaCl in buffer C. The enzyme eluted as a single peak of activity (Fig. 1) at 250 mM NaCl with a recovery of 70% of the input activity. Pooled samples were concentrated, dialyzed against buffer C plus 10% polyethylene glycol 6000, glycerol was added to 50% final concentration, and the samples were stored at –20°C.

Deletion strains

MG1655 Δ *truD*::kan was constructed by a modification of the gene replacement method of Datsenko and Wanner (2000). A deletion allele containing the amino- and carboxy-terminal fragments of *truD* linked to a kanamycin resistance cassette was constructed by PCR amplification from plasmid pUC4K (Amersham). The 5' primer (5'-GCGGTGGCGCTACTCATTAAAGGCAACA AAATGATTGAGGATTACGAATTCCCCGGATC-3') was the *truD* sequence of residues –29 to +9 (uppercase), in which the A of the initiating AUG (italics) is +1 followed by the complement of residues 1689–1670 of pUC4K (underlined). The 3' primer (5'-CCATTCGCCACTGCCTGGCAATGCGGCGGTTATCATCC AATTCTGATTAGAAAACTCATC-3') was the complement of *truD* residues +795 to +759 (uppercase and the first C residue underlined), followed by the sequence of residues 710–731 of pUC4K (underlined). After PCR amplification of the allele, the wild-type *truD* gene was replaced with the deletion allele as described (Datsenko and Wanner 2000).

Backcross of Δ *truD* to wild type by P1-mediated transduction

Backcrosses mediated by bacteriophage P1 were performed according to Davis et al (1980). For preparation of P1 lysate, 50 μ L of a fresh overnight culture of the Δ *truD* strain was diluted in 5 mL of LB, 0.2% glucose, 25 mM CaCl₂, and grown for 30 min at 37°C. After addition of bacteriophage P1 to a final concentration of 1 $\times$ 10⁷ plaque forming units/milliliters, the cultures were grown for an additional 4 h. Cell debris was removed by centrifugation, 100 μ L of chloroform was added to the supernatant, and the mixture stored at 4°C. For P1-mediated transductional backcross, 100 μ L of a fresh overnight culture of *E. coli* MG1655 was added to 5 mL of LB and grown to an A₆₀₀ of ~0.3 at 37°C. Cells were harvested by centrifugation and resuspended in 2.5 mL of 5 mM CaCl₂, 10 mM MgSO₄. A total of 100 μ L of P1 lysate was added to an equal volume of the cells, and incubated at 30°C for 30 min. After adding 150 μ L of 1 M sodium citrate, cells were grown an additional 1.5 h to allow expression of drug resistance. The cells were then plated on LB plus 0.04 mg/mL kanamycin plates. Cotransduction frequency was converted to kilobase by the Wu formula (Bachman et al. 1976).

Overexpression of TruD and affinity purification

A total of 0.2 mL of overnight cultures of BL21(DE3) cells with appropriate pET28a constructs; pET28a::*truD*(D80D), pET28a::*truD*(D80N), and pET28a::*truD*(D80T), were diluted in 20 mL of LB plus 0.04 mg/mL kanamycin. Cells were grown to an A₆₀₀ of 0.6 at 37°C in a shaker, IPTG was added at a final concentration of 2 mM, and cells were induced for 3 h. The cells were harvested by centrifugation at 4°C in a Sorvall SS-34 rotor at 10,000g for 10 min, resuspended in 5 mL of ice-cold 1X Binding Buffer (Novagen pET System Manual, 10th edition), and lysed by passage through a French press at 18,000 psi (1260 bar) four times. Cell debris was removed by centrifugation in a Sorvall SS-34 rotor at 4°C at 14,000g for 20 min. The post-centrifugation supernatant was filtered through a 0.45 micron syringe-end filter (Nalgene). Wild-type and D80 mutants of TruD were affinity purified using

His-Bind Quick 900 Cartridges (Novagen) following the protocol of the manufacturer (Novagen TB054, Rev.B., 0602).

Pseudouridine sequencing

Sequencing of tRNA^{Glu} with ³²P end-labeled primers was done as described (Ofengand et al. 2001a).

Other methods and materials

SDS gel electrophoresis was performed as described previously (Raychaudhuri et al. 1998). For amino-terminal sequencing of TruD, the protein was transferred from SDS gels as in Figure 1B to a PVDF membrane, and amino-terminal sequencing performed by automated Edman degradation on an Applied Biosystems Model 491 Protein Sequencer (Procise) at the University of Miami Protein Analysis Core Facility. Exponential growth rates and competition studies were done as described previously (Gutgsell et al. 2000). Transformation, growth of transformed cells, and isolation of total RNA were performed as described (Del Campo et al. 2001). Protein was determined by the Bradford assay.

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