RNA (1995), *1*:102-112. Cambridge University Press. Printed in the USA.

Purification, cloning, and properties of the tRNA Ψ55 synthase from *Escherichia coli* *

KELVIN NURSE,[1] JAN WRZESINSKI,[1] ANDREY BAKIN,[1] BYRON G. LANE,[2] and JAMES OFENGAND[1]

[1] Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110, USA
[2] Department of Biochemistry, University of Toronto, Toronto, Ontario, M5S 1A8, Canada

ABSTRACT

tRNA pseudouridine 55 (Ψ55) synthase, the enzyme that is specific for the conversion of U55 to Ψ55 in the $m^5U\Psi CG$ loop in most tRNAs, has been purified from *Escherichia coli* and cloned. On SDS gels, a single polypeptide chain with a mass of 39.7 kDa was found. The gene is a previously described open reading frame, P35, located at 68.86 min on the *E. coli* chromosome between the *infB* and *rpsO* genes. The proposed name for this gene is *truB*. There is very little protein sequence homology between the *truB* gene product and the *hisT* (*truA*) product, which forms Ψ in the anticodon arm of tRNAs. However, there was high homology with a fragment of a *Bacillus subtilis* gene that may produce the analogous enzyme in that species. The cloned gene was fused to a 5′-leader coding for a $(His)_6$ tract, and the protein was overexpressed >400-fold in *E. coli*. The recombinant protein was purified to homogeneity in one step from a crude cell extract by affinity chromatography using a Ni^{2+}-containing matrix. The SDS mass of the recombinant protein was 41.5 kDa, whereas that calculated from the gene was 37.3. The recombinant protein was specific for U55 in tRNA transcripts and reacted neither at other sites for Ψ in such transcripts nor with transcripts of 16S or 23S ribosomal RNA or subfragments. The enzyme did not require either a renatured RNA structure or Mg^{2+}, and prior formation of m^5U was not required. Stoichiometric formation of Ψ occurred with no requirement for an external source of energy, indicating that Ψ synthesis is thermodynamically favored.

Keywords: *Bacillus subtilis* gene; chemical sequencing; $(His)_6$ leader; modified base; pseudouridine; TΨCG

INTRODUCTION

Pseudouridine (Ψ), the 5-ribosyl isomer of uridine, has been known for almost 35 years (Davis & Allen, 1957; Cohn, 1959, 1960; Scannell et al., 1959; Yu & Allen, 1959), and its location in most tRNAs in a common pentanucleotide sequence, $Gm^5U\Psi CG$ (Zamir et al., 1965), is also well known. Two alternate mechanisms of Ψ biosynthesis have been considered (Goldwasser & Heinrikson, 1966). In the first, ΨTP was proposed to be formed and subsequently incorporated into polynucleotide, the signal for ΨTP incorporation being some unknown rare DNA base. In the second, the conversion of U to Ψ took place at the polynucleotide level. This latter hypothesis was proven by subsequent work, which showed clearly the direct conversion of U residues in a polynucleotide into Ψ without an intermediate stage of breakdown and resynthesis (Johnson & Söll, 1970; Ciampi et al., 1977). In this reaction, the glycosyl bond to N_1 of the pyrimidine ring is broken and replaced by a carbon–carbon link to C_5 without the need for an external source of energy. The mechanism of this reaction is unknown, although it has been proposed that, after cleavage of the C–N glycosyl bond, a simple rotation around the N_3–C_6 axis of the uracil ring while still on the enzyme surface brings the C_5 atom into the proper spatial orientation with respect to the C_1' atom of the ribose moiety and allows bond formation (cited in Goldwasser & Heinrikson, 1966).

Despite early attempts to purify and characterize the enzymes that form Ψ in tRNA, the only Ψ synthase that has been cloned up to now is one from *Escherichia coli* that forms Ψ in the anticodon loop and stem (Arps et al., 1985; Kammen et al., 1988). This enzyme does not form Ψ55, the most common of all Ψ residues in tRNA. That activity had earlier been shown to reside

* This paper is dedicated to Helga and Walter Kersten, University of Erlangen, Erlangen, Germany, on the occasion of their retirement from the University.

Reprint requests to: James Ofengand, Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110, USA.

in a different protein (Schaefer et al., 1973; Ciampi et al., 1977). Evidence for three distinct tRNA Ψ synthases in yeast has been obtained (Samuelsson & Olsson, 1990)—one for Ψ55, one for the anticodon arm region, and one for the dihydrouridine-containing region—but none of these distinct activities has been purified to homogeneity and cloned.

In this report, we describe the purification, cloning, and partial characterization of the tRNA Ψ55 synthase from *E. coli*, compare its sequence with that of the other tRNA Ψ synthase whose gene is known, and locate a partial gene sequence in *Bacillus subtilis* that is probably the tRNA Ψ55 synthase in that organism.

RESULTS

Purification of the tRNAΨ55 synthase

The detection of the enzyme was a serendipitous outgrowth of a search for the enzyme that forms Ψ516 in *E. coli* 16S RNA. Having located such an activity in fractions eluted from a DEAE column by assay with a suitable substrate (J. Wrzesinski, unpubl. results), the same region of the column was probed with a $tRNA^{Val}$ transcript that was intended as a negative control. In both cases, the enzyme was assayed by measuring the release of ^3H from the 5-position of uridine as uridine is converted into pseudouridine (Cortese et al., 1974), the labeled uridine having been incorporated into an appropriate polynucleotide by in vitro transcription. Unexpectedly, a strong activity distinct from the Ψ516 synthase was found. This activity was shown to release a maximum of 1 mole of ^3H per mole of tRNA transcript (see also Fig. 2). Although it was not known at the time if this was indeed a Ψ synthase, subsequent analysis proved this to be the case. Three further column chromatography steps were employed to purify the enzyme, namely MonoS (pH 6.8), MonoS (pH 7.8), and MonoQ. In each case, only a single peak of activity was found. The activity profile of the last column is shown in the upper panel of Figure 1 and the SDS gel analysis in the lower one. There is only one well-defined protein band in the fractions, and its intensity varies according to the activity profile. Thus, this polypeptide probably corresponds to the enzyme.

Characterization of the enzymatic activity

There are several enzymatic activities in *E. coli* capable of releasing ^3H from the C5 of uracil in addition to Ψ synthases. Probably the most important one is RUMT (EC 2.1.1.35), the *trmA* gene product. This is the enzyme that methylates C5 of U54 in tRNA. Other enzymes that derivatize the C5 position should also release a proton, such as the enzymes that form cmo^5U, mnm^5U, etc. The first indication that this activity forms Ψ55 was the kinetics of ^3H release with tRNA transcripts (Fig. 2A). With both a $tRNA^{Val}$ and a $tRNA^{Phe}$ transcript, approximately 1 mole of ^3H was released per mole of transcript and with identical kinetics. The only C5-modified uracils common to both sequences are m^5U54 and Ψ55. Sequencing analysis of the modified $tRNA^{Val}$ transcript by the method of Peattie (1979) showed clearly that U55 had been converted into Ψ, whereas U54 was not affected (Fig. 2B). In this method, 3′-pCp-labeled RNA is treated with hydrazine and aniline to induce chain breaks at all U residues, which show up as shorter oligomers upon electrophoresis. m^5U and Ψ, being resistant to hydrazinolysis, do not produce breaks, and bands are missing at those sites. In the native RNA, bands are absent at U54 and U55, although strong bands are present at all other U sites. In the unmodified transcript, these U residues also appear as strong bands; in addition, U54 and U55 are

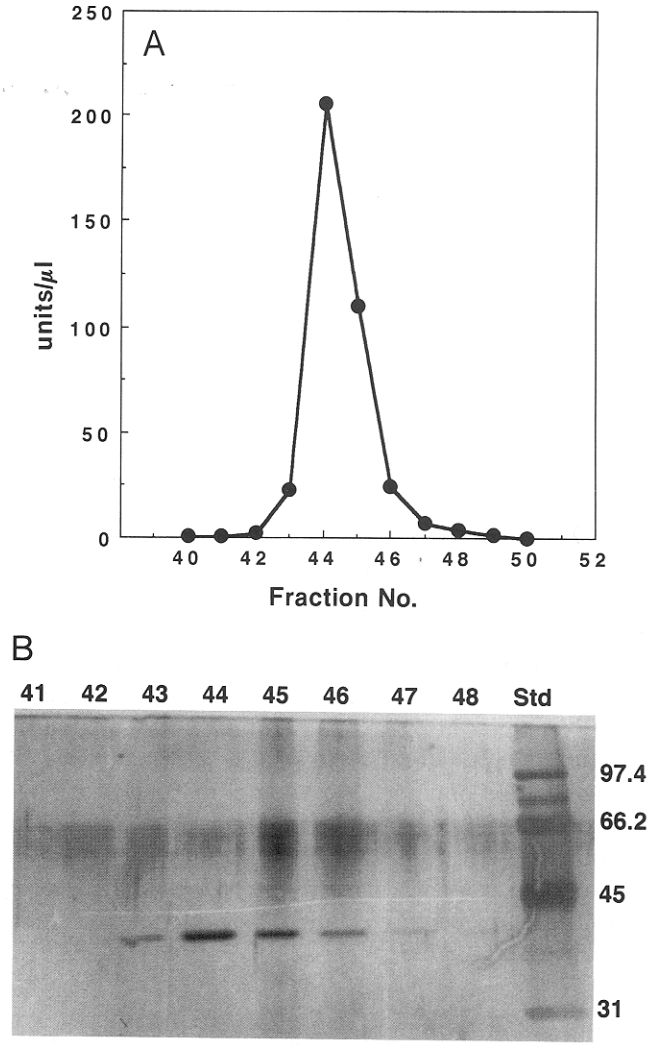

FIGURE 1. Final step of purification of tRNA Ψ55 synthase on Mono Q. **A:** Chromatography of the enzyme on an FPLC Mono Q column was done as described in the Materials and methods section. **B:** Column fractions as indicated were electrophoresed in SDS gels. The MW values of the standards are indicated.

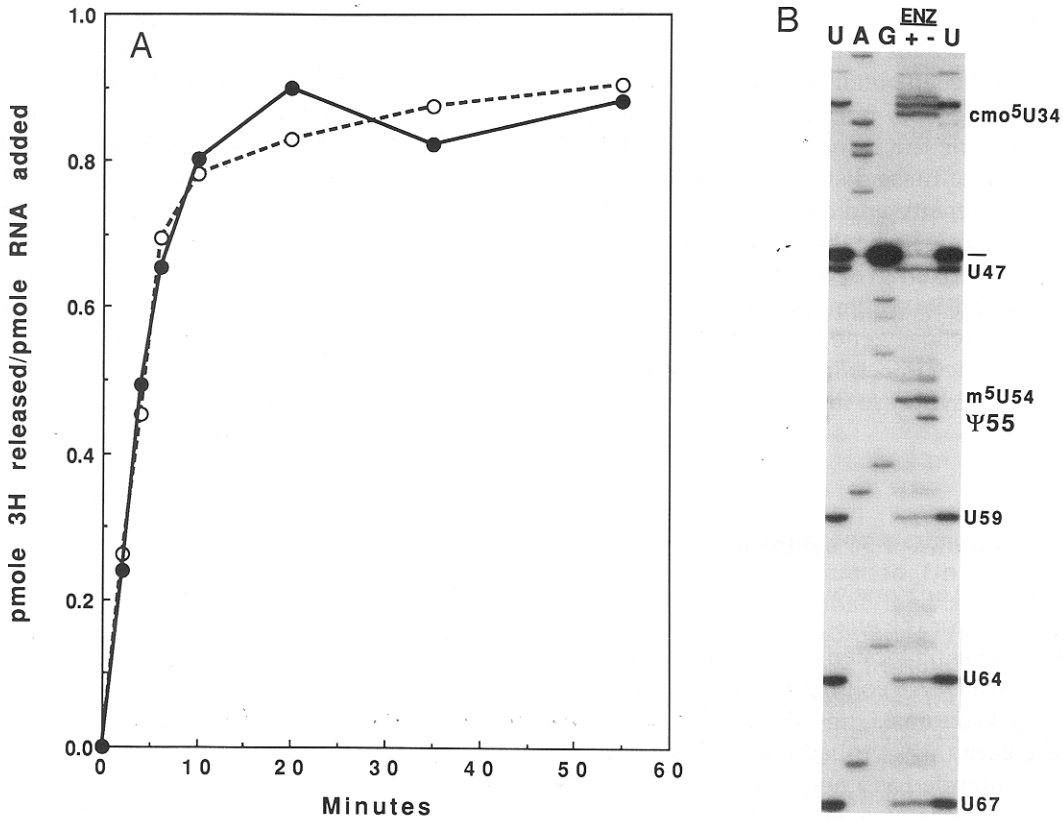

FIGURE 2. Identification of the enzyme as a Ψ55 synthase. **A:** Kinetics of ^{3}H release from tRNA transcripts. [5-^{3}H]uracil-containing transcripts of tRNA^{Val} (○) and tRNA^{Phe} (●) were prepared as described in the Materials and methods section. Pseudouridine formation was measured as release of ^{3}H as described in the Materials and methods section. **B:** Site of formation of Ψ in the tRNA^{Val} transcript. U, A, and G, 3′-end labeling with [5′-^{32}P]pCp, and chemical sequencing were as described by Peattie (1979) using native tRNA^{Val}. Enz + was a [5-^{3}H]uracil-containing tRNA^{Val} transcript treated with an amount of enzyme sufficient to yield a plateau amount of ^{3}H release corresponding to 1.0 mol/mol of RNA. Enz − was a control sample lacking enzyme. Both tRNAs were labeled with pCp and sequenced for U. The U and U-derived residues are indicated. The horizontal line shows the position of m^{7}G46.

clearly visible. After treatment with the enzyme, the only detectable change was the almost complete disappearance of the band at U55 but not at U54, demonstrating conclusively that the enzyme converts U55 into Ψ55. Moreover, the cloning of the enzyme (see below) shows that it is not RUMT. Note also the strong stop corresponding to m^{7}G, indicated by the bar, in the native RNA lanes, because this base is also cleaved by the U sequencing procedure, and the absence of cleavage at the position of cmo^{5}U34. Apparently this base is resistant under these conditions, like m^{5}U, although it was reported by others (Lankat-Buttgereit et al., 1987) to be cleaved like U. Treatment of a tRNA^{Phe} transcript also showed conversion of U55 but not U54 (data not shown).

Identification of the gene

N-terminal amino acid sequencing of the gel-purified protein band shown in Figure 1 yielded the sequence underlined in Figure 3. A search of the GenBank database located a putative ORF containing this sequence (Fig. 3). Although there was an exact match for the entire 15 residues sequenced, the first 5 codons of the ORF were not represented in the amino acid sequence. This could be explained by an inadvertent cleavage of the polypeptide used for sequencing between the fifth and sixth residues. This proved fortuitous, because four other attempts at sequencing even larger quantities of more highly purified fractions did not yield an unequivocal sequence, possibly because the true N-terminus was blocked. In all four instances, there was an indication for the presence of serine at the N-terminus, but methionine was never detected. We suggest, therefore, that the natural protein may have an N-blocked serine at the N-terminus.

The gene is a previously described ORF, P35, found at approximately 68.86 min on the *E. coli* chromosome between *infB* and *rpsO* (Sands et al., 1988). The gene codes for a strong Shine–Dalgarno sequence 4 nt from the initiating AUG. As noted by Sands et al. (1988), the initiation codon for P35 overlaps the termination codon of the ORF immediately upstream, P15B, and the two genes appear to be transcriptionally coupled.

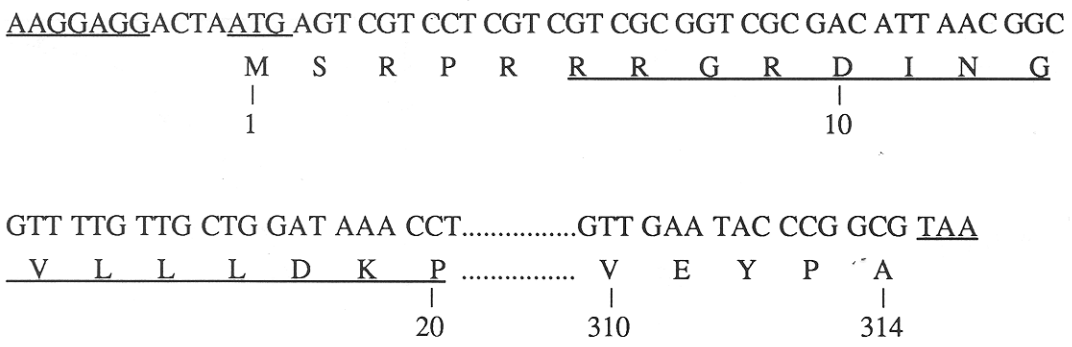

FIGURE 3. Gene sequence of tRNA Ψ55 synthase. The upper line shows the gene sequence, and the deduced amino acid sequence is below. In the nucleotide sequence, the Shine–Dalgarno, initiation codon, and termination codon are underlined. The amino acid sequence underlined was obtained by experimental analysis of the purified protein. The total number of amino acid residues calculated from the ORF is indicated.

The calculated MW is 35,091, and that determined experimentally was 39.7 ± 0.4 kDa as an average of four determinations (see, for example, Fig. 1B and Fig. 5, lanes 4 and 5).

Cloning of the gene and overexpression of ^{3}H release activity

The P35 ORF was cloned into pET-15b by standard PCR methods. Two clones, T2 and T7, were obtained with the correct insert. As shown in Figure 4, induced cells containing these clones produced a large amount of a protein about 40 kDa in size (lanes 2 and 3), whereas no such band was visible in the pET control (lane 1). The overexpressed protein remained soluble and was found in the S15 supernatant fraction (lanes 5 and 7), whereas almost none was in the S15 pellet (lanes 9 and 10). The S15 supernatant extract from clones T2 and T7, but not from pET, contained very high amounts of ^{3}H release activity, from 400 to over 2,000 times that in the control extract (Table 1). The true increase is no doubt higher, because the S15 extract should also contain RUMT activity as well as other ^{3}H release activities. Possibly the sixfold difference in pET-15b activity in the two preparations is due to a preferential loss of one or more of these other activities during the workup. That this large increase in activity is indeed a result of overexpression of the P35 ORF is shown below.

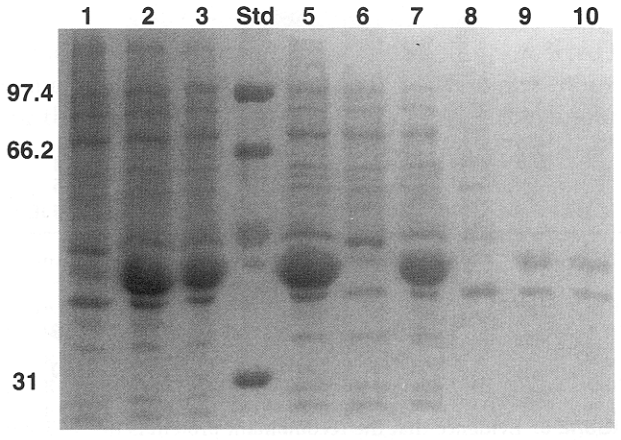

FIGURE 4. Overexpression of tRNA Ψ55 synthase gene product and cellular localization of the protein. Cells were transformed with the indicated plasmid, induced, harvested, and lysed as described in the Materials and methods section. Lanes 1–3, cells broken by boiling in SDS; lanes 5–10, cells broken by sonication in 1/8 the original volume and fractionated into an S15 supernatant and a pellet. Aliquots from approximately equivalent amounts of cells were loaded in each lane. Lanes 1, 6, 8, pET vector lacking the gene insert; lanes 2, 5, 9, clone 2; lanes 3, 7, 10, clone 7. Lanes 5–7, S15 supernatant; lanes 8–10, solubilized S15 pellet; lane 4, MW standards.

Enzymatic activity of the affinity-purified recombinant protein

The P35 ORF gene was cloned such that its translation product should contain a $(His)_6$ sequence as part of a leader peptide. This allowed affinity purification of the protein specified by the cloned gene on a Ni^{2+}-containing resin column that is specific for polyhistidine tracts. Ψ synthase activity in such an affinity-purified protein would constitute definitive proof that the gene cloned is the gene for Ψ synthase. Whereas before puri-

TABLE 1. Ψ synthase activity of induced cell extracts with and without the P35 ORF insert.[a]

Source	Preparation 1	Preparation 2
pET-15b	0.06	0.01
Clone T2	28.7	22.6
Clone T7	22.2	–

[a] Values are expressed in (units enzyme/mL of extract) × 10^{-6}. S15 cell extract was prepared as described in the Materials and methods section from induced cells containing the indicated plasmid. The extract was assayed for Ψ formation by the ^{3}H release assay.

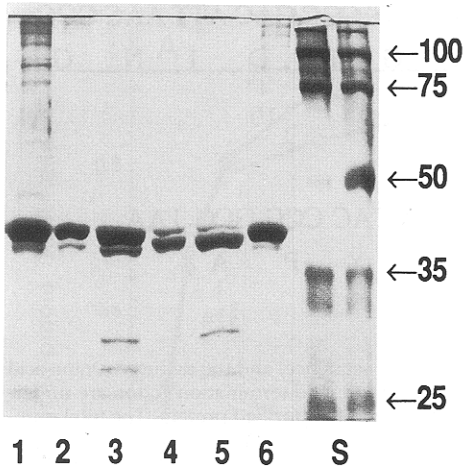

FIGURE 5. Removal of the $(His)_6$-containing tag from the affinity-purified recombinant protein by thrombin cleavage. Recombinant synthase was digested with thrombin at 0.2 mg/mL synthase and 0.2 units/mL thrombin. At 60 min, a second 0.2 units/mL thrombin was added. Lane 1, 8 μL of the reaction mixture without thrombin at 0 time; lanes 2 and 3, 4 and 8 μL, respectively, of the reaction mixture without thrombin after 120 min incubation; lanes 4 and 5, as lanes 2 and 3 but with thrombin; lane 6, untreated affinity-purified recombinant synthase; S, molecular weight standards (Novagen) whose values are as indicated.

fication the S15 extract contained many proteins (Fig. 4, lanes 5 and 7), after elution from the column, essentially only a single protein band was detected (Fig. 5, lane 6) with a monomer MW of 41.5 kDa. Because the 5′-tag increases the MW by 2,179 Da, the expected value for the recombinant protein, 37.3 kDa, is in reasonable agreement with that found experimentally.

The Ψ synthase enzymatic activity resides in this affinity-purified protein. Treatment of [5-^{3}H]uracil-containing $tRNA^{Val}$ and $tRNA^{Phe}$ transcripts with this protein yielded the same rate and yield of ^{3}H release, with a plateau value of approximately 1 mole released per mole of RNA (Fig. 6A). This result is the same as that obtained in Figure 2A with the natural enzyme. To confirm that Ψ55 was being formed, chemical sequencing was performed with both tRNA transcripts (Fig. 6B). The results show clearly that Ψ55 was formed in both cases. U54 was not affected and *only* Ψ55 was formed. U32 and U39, which are Ψ in natural $tRNA^{Phe}$, were not converted to Ψ.

Requirements and specificity of the recombinant enzyme

The standard assay for this enzyme included both a denaturation–renaturation step for the substrate and inclusion of Mg^{2+}. However, it was shown earlier for the *E. coli* anticodon arm Ψ synthase that Mg^{2+} was not required (Green et al., 1982), and this is also true for two rRNA Ψ synthases that were purified in our laboratory (J. Wrzesinski & K. Nurse, unpubl. results). Therefore, the dependence of the Ψ55 enzyme on these

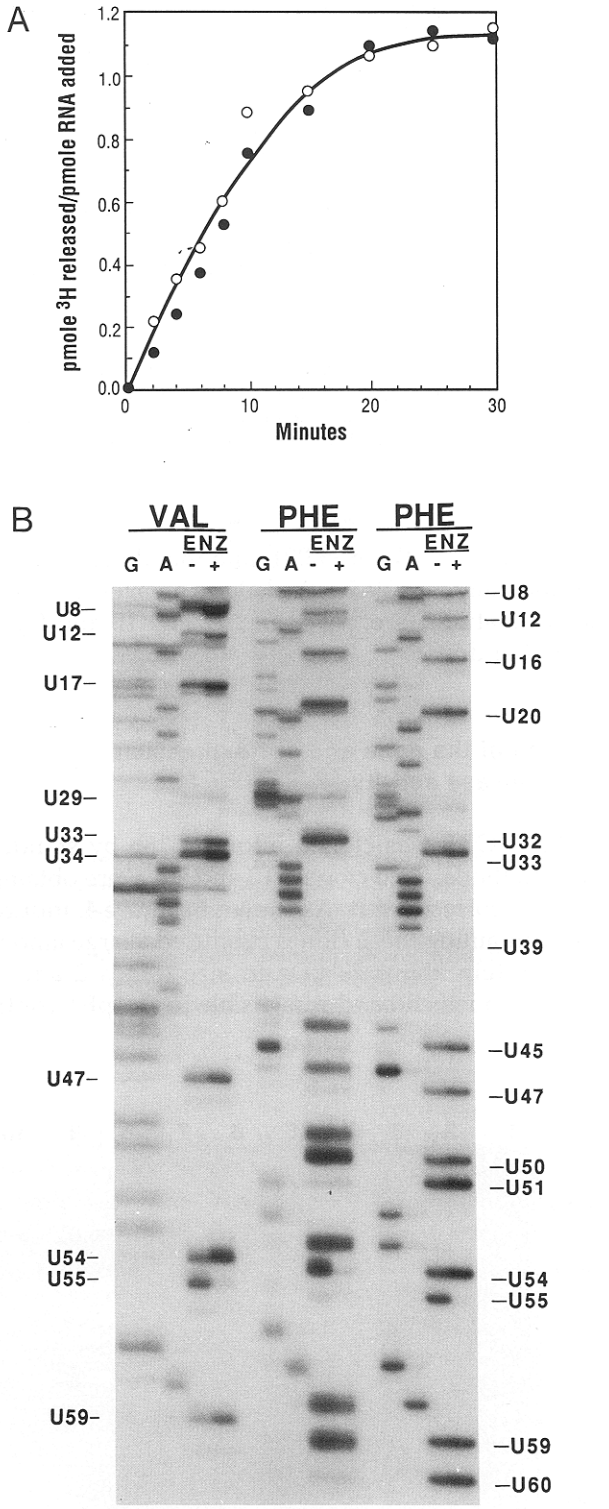

FIGURE 6. Evidence that the recombinant protein is the Ψ55 synthase. **A:** Kinetics of ^{3}H release from $tRNA^{Val}$ and $tRNA^{Phe}$ transcripts. ○, $tRNA^{Val}$ transcript; ●, $tRNA^{Phe}$ transcript. Reactions for Ψ formation were as described in the Materials and methods section. **B:** Site of Ψ formation in $tRNA^{Val}$ and $tRNA^{Phe}$ transcripts. Methodology was as described in Figure 2B except that tRNA transcripts were used for the sequencing lanes. The plateau ^{3}H release values for $tRNA^{Val}$ and $tRNA^{Phe}$ transcripts were 1.1 and 1.0 mol/mol RNA, respectively. The A, G, +, and − lanes are as described in Figure 2B. The center Phe lanes were obtained using a transcript with one more residue at the 3′-end than was used in the Phe lanes on the right.

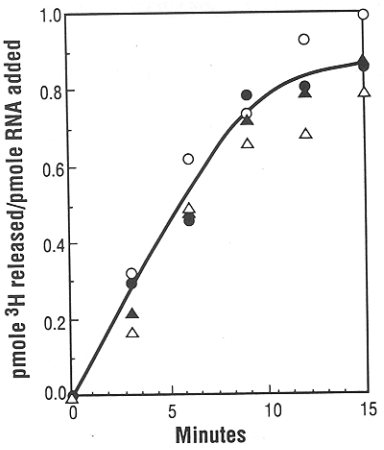

FIGURE 7. Effect of Mg, EDTA, and denaturation–renaturation of the tRNA transcript on its ability to act as a substrate. ●, Ψ formation was assayed as described in the Materials and methods section; ○, the denaturation–renaturation cycle was omitted; ▲, Mg^{2+} was replaced by 0.1 mM EDTA; △, Mg^{2+} was replaced by 0.1 mM EDTA and the denaturation–renaturation cycle was omitted as well.

two parameters was examined. As shown in Figure 7, the rate and yield of Ψ formation were the same whether the substrate was heat-treated or not, and whether Mg^{2+} or EDTA was present. Because heating to 58 °C for 5 min in the presence of 0.1 mM EDTA had no effect, it seems likely that the enzyme recognizes only the local sequence environment around Ψ55, rather than a more complex secondary or tertiary structure. However, this point needs to be tested more rigorously, for example by using appropriate tRNA fragments as substrate.

RNA specificity was tested with five different lengths of ribosomal RNA transcripts from both subunits. None was able to react with the enzyme either with or without Mg^{2+}, whereas the tRNA transcript reacted to completion (Table 2). The time chosen was just enough for the reaction to reach completion with the amount of enzyme used (see Fig. 7). We conclude that this enzyme is completely specific for formation of Ψ at position 55 in tRNAs.

TABLE 2. Specificity of tRNA Ψ55 synthase for different RNAs.[a]

Substrate	+ Mg^{2+}	+ EDTA
$tRNA^{Val}$	1.05	1.10
16S RNA (1–1542)	0.02	<0.01
16S RNA (1–526)	<0.01	0.01
16S RNA (1–678)	<0.01	<0.01
23S RNA (1–2904)	<0.01	<0.01
23S RNA (1–847)	<0.01	<0.01

[a] Values are expressed in mole ^{3}H released/mole RNA added. [5-^{3}H]uracil-containing transcripts were prepared as described in the Materials and methods section. The rRNA fragments are designated by their beginning and ending nucleotide. Reaction was measured in the standard assay at 37 °C for 15 min except that the pretreatment at 58 °C was omitted. When EDTA (1 mM) was present, Mg^{2+} was omitted. The maximum Mg^{2+} contribution from the RNA samples was 0.1 mM. The RNAs were all assayed at 300 nM with the recombinant enzyme containing the N-terminal tag.

Effect of the N-terminal tag on enzymatic activity

The recombinant enzyme with the N-terminal tag of 20 amino acids had the same specificity as the natural enzyme (Fig. 6B; Table 2), but its effect on the kinetics of the reaction was unknown. In order to test this, 17 of the 20 amino acids of the tag were removed by digestion at its thrombin site. After thrombin treatment, the enzyme was assayed without further purification. In this experiment, performed as described in the legend for Figure 5, aliquots were taken at 30-min intervals up to 2 h and assayed immediately after sampling. There was no progressive change with time either with or without thrombin. The average specific activity of the recombinant enzyme was 12.2×10^6 units/mg protein after incubation in the presence of thrombin and 11.2×10^6 after incubation in its absence, for a before/after ratio of 0.92. Gel electrophoresis (Fig. 5) confirmed that cleavage was almost complete after 2 h. Thus, there is no measurable effect of the 5′-leader on enzymatic activity.

Comparison of the amino acid sequence with other known sequences

A search of the GenBank database revealed the existence of a fragment of a gene in *B. subtilis*, also in the *infB* operon, whose deduced amino acid sequence had a pronounced similarity to this enzyme (Fig. 8). This gene has a strong Shine–Dalgarno sequence that is suitably positioned from the initiating AUG (not shown). Its amino acid sequence has a 49% identity with the tRNA Ψ55 synthase and a 69% similarity over the N-terminal 65-amino acid sequence available. We suggest that it is the equivalent *B. subtilis* enzyme, and we are attempting to obtain the remainder of the sequence in order to verify this by overexpression of the gene in *E. coli*.

Figure 8 also shows a comparison of this enzyme (EcY55) with the only other known Ψ synthase, the *hisT* gene (EcHisT) product that converts U residues in the anticodon arm of tRNA into Ψ. Although BESTFIT analysis gives a 22% identity and a 47% similarity over 293 amino acids, examination of Figure 8 shows a much greater dispersion of identical amino acids than was seen with the BsY55? sequence; in addition, there are 13 gaps in this comparison.

DISCUSSION

Substrate specificity

There are multiple tRNA Ψ-forming enzymes, each one specific for a region of the tRNA in which Ψ occurs

```
BsY55?                1 MVNGVLLLHKPVGMTSHDCVMKIRKLLKTKKVGHTGTL 38
                          :|||||.|| ||.|:|.: |::::.....:.||||.|
EcY55          1 MSRPRRRGRDINGVLLLDKPQGMSSNDALQKVKRIYNANRAGHTGAL 47

EcHisT                                                    1 MS 2


BsY55?    39 DPEVSGVLPICVG...RATKIVEYLTEKSK 65
             || ..|:||||:|     |||: :|| :..|
EcY55     48 DPLATGMLPICLG...EATKFSQYLLDSDKRYRVIARLGQRTDTSDADGQ 94
             |. ...:..|.||    ::.|: .:  :.: | .| .:|:.       | :|
EcHisT     3 DQQQPPVYKIALGIEYDGSKYYGWQRQNEVR.SVQEKLEK......ALSQ 45


EcY55     95 IVEERPVTFSAEQLAAALDTFRGDIEQIPSMYSALKYQGKKLYEYARQGI 144
             :.:|. ..|:|:. .|::    :|. : :.    .||: ::
EcHisT    46 VANEPITVFCAGRTDAGV...HGTGQVVHFETTALRKDA.......... 81


EcY55    145 EVPREARPITVYELLFIRHEGNELELEIHCSKGTYIRTIIDDLGEKLGCG 194
                  |:.:.|          ..:|. :| ..   :::|: ||: .:::..
EcHisT    82 .....AWTLGV.........NANLPGDIAVR...WVKTVPDDFHARFSAT 114


EcY55    195 AH....VIYLRRLAVSKYPVERMVTLEHLR.ELVEQAEQQDIPAAELLDP 239
             |:     :|| :|| .. .. :     .|.|  | :..|.|  :...:: .
EcHisT   115 ARRYRYIIYNHRLRPAVLSKGVTHFYEPLDAERMHRAAQCLLGENDFTSF 164


EcY55    240 LLMPMDSPA.............SDYPVVNLPLTSSVYFKNGNPV...RTS 273
               :. :|..               :.|.||::. .. |. .  | |   ..
EcHisT   165 RAVQCQSRTPWRNVMHINVTRHGPYVVVDIKANAFVHHMVRNIVGSLMEV 214


EcY55    274 GAPLEGLVRVTE..GENGKFIGMGEIDDEGRVAPRRLVVEYPA 314
             ||. :.  ::.|  :..:: :: :. ..||      :.|:||.
EcHisT   215 GAHNQPESWIAELLAAKDRTLAAATAKAEGLYL...VAVDYPDRYDLPKP 261


EcHisT   262 PMGPLFLAD 270
```

FIGURE 8. Sequence comparison of the Ψ55 synthase with other related proteins. The sequences of the tRNA Ψ55 synthase (EcY55), the anticodon arm Ψ synthase (EcHisT), and an ORF in *B. subtilis* (BsY55?) that has similarity to EcY55 were compared using the BESTFIT module of the GCG Sequence Analysis Software suite of programs (Devereux et al., 1984). The bar, colon, and period indicate identity, high similarity, and low similarity, respectively, as defined by the program. The numbers at the left and right margins are amino acid sequence numbers.

(Singer et al., 1972; Schaefer et al., 1973; Cortese et al., 1974; Ciampi et al., 1977; Green et al., 1982; Samuelsson & Olsson, 1990; Szweykowska-Kulinska et al., 1994). There is also evidence for multiple specific Ψ synthases for snRNAs (Patton, 1994; Patton et al., 1994) and for ribosomal RNAs (J.W., A.B., K.N., B.G.L., & J.O., unpubl. results). The enzymatic activity capable of specifically forming Ψ55 has been described in *E. coli* (Schaefer et al., 1973) and *Salmonella typhimurium* (Ciampi et al., 1977), but until now the enzyme had not been purified or cloned. In this work, we have described both the purification of the enzyme and its location in the *E. coli* genome.

The substrate specificity of the cloned enzyme was shown in several ways. First, given a $tRNA^{Val}$ and a $tRNA^{Phe}$ transcript, the enzyme only formed Ψ55, although there were two other sites available in the $tRNA^{Phe}$ transcript that normally contain Ψ in mature tRNA. Second, several other RNAs did not react (Table 2), even though they were assayed under the same conditions as the tRNA transcripts. Third, the enzyme did not require added Mg^{2+} (Fig. 7; Table 2) and thus

probably has no requirement for higher-order structure in the RNA substrate. It may well be that only the UUCG loop and closing base pairs would be needed, but this has not yet been tested. Clearly, prior formation of m^5U is not required.

Gene and protein sequence

The gene was identified as a previously sequenced ORF, P35, located between the *infB* and *rpsO* genes. We propose the name *truB* for this gene (tRNA pseudo U formation or U modification), and that the *hisT* gene be renamed *truA*. Despite the fact that both enzymes should have the same catalytic center, no obvious sequence elements could be found that corresponded in both sequences (Fig. 8). Perhaps when more Ψ synthases are cloned, some elements of identity will emerge. On the other hand, a potentially equivalent enzyme was detected in the genome of *B. subtilis*.

Kammen et al. (1988) noted two internal repeats, RAXQC, involving two of the three cysteine residues of the *hisT* (*truA*) protein, and postulated that they might make up part of the catalytic center because intact SH groups had been shown to be essential for the maintenance of an active enzyme. There are three cysteine residues in the *truB* protein sequence, but there is no internal sequence match involving any two of the cysteine residues, nor do any fit the RAXQC motif. Thus, it is not likely that this proposal will be generally valid.

The *truB* sequence does not use the AGA/G pair of codons for arginine, despite the presence of 27 Arg residues among 314 total amino acids. The use of these rare codons, particularly within the first 25 codons, is a characteristic of a group of genes essential for various cellular functions that appear to be globally regulated by the availability of the corresponding $tRNA^{Arg}$ (Chen & Inouye, 1994). If the *truB* gene is essential, it is not regulated in this manner.

Requirement for the enzyme

Because almost all tRNA species contain Ψ55, it might be supposed that this modification is essential for the cell. However, no specific function has yet to be shown to be dependent on the presence of Ψ in tRNA (references cited in Bakin & Ofengand, 1993). Inactivating mutations in the *hisT* (*truA*) gene were shown to have little effect (Chang et al., 1971), although a role in the regulation of some processes has been described (Johnston et al., 1980; Tsui et al., 1991). Preliminary evidence for the lack of an essential role for the *truB* gene was described by Sands et al. (1988). Rigorous exclusion of the gene product was not done, however. It will be necessary to delete at least part of the *truB* gene in order to demonstrate that synthesis, processing, and function of tRNAs proceed in the absence of the ubiquitous Ψ55. Gene disruption studies are currently under way in our laboratory.

Energetics of Ψ formation

The conversion of U to Ψ requires no external energy source and goes virtually to completion. The extent of reaction cannot be determined by the ^3H exchange assay because any resynthesis of U that re-incorporates a proton would draw it from the solvent, in which the released ^3H is highly diluted. However, direct measurements using unmodified in vitro RNA transcripts have shown stoichiometric formation of Ψ (Szweykowska-Kulinska et al., 1994). The same conclusion can be drawn from the sequencing gels shown in Figures 2 and 6, which show that after reaction of transcripts with the synthase, there is no evidence for any remaining U55. Because even a small percentage of U would generate a band on the gel, this assay is quite sensitive for detection of residual U. The energetic considerations that place the U to Ψ equilibrium far to the side of Ψ do not appear to have been discussed previously, perhaps because in the absence of a purified enzyme and substrate, the existing data were not considered sufficiently reliable. Now, however, it seems clear that it is the case. The difference in p*K* values between Ψ_C, the natural isomer, of 8.97 (Ofengand & Schaefer, 1965) and the uridine p*K* of 9.25 (Fox & Shugar, 1952) would not appear sufficient to drive the reaction. This aspect needs further study.

MATERIALS AND METHODS

Materials

[5-^{3}H]UTP and [5′-^{32}P]pCp were from Amersham. $tRNA^{Val}$ and $tRNA^{Phe}$ came from Subriden RNA. RNasin was from Promega. Restriction enzymes, acetylated bovine serum albumin, and T4 RNA ligase came from New England Biolabs. T7 RNA polymerase was from Ambion, Inc. Plasmid pET-15b, the BL21/DE3 and Novablue strains of *E. coli*, His-Bind resin, and thrombin were obtained from Novagen, Inc. T4 DNA ligase and shrimp phosphatase were from U.S. Biochemical. Nuclease-free bovine serum albumin came from BRL. Yeast inorganic pyrophosphatase, Norit A washed with HCl, and polyethylene glycol 6000 were from Sigma. Hydrazine (#21,515-5), dimethyl sulfate (#D18,630-9), and aniline (#24,228-4) were from Aldrich, and diethylpyrocarbonate (#D-5758) came from Sigma. Deoxyoligonucleotide primers were prepared as described previously (Bakin & Ofengand, 1993). DEAE Sepharose CL6B, and MonoS and MonoQ FPLC columns were from Pharmacia. Protein standards came from Bio-Rad (#161-0304) or Novagen, Inc. (#69149-1).

Buffers

Buffer compositions were as follows: Buffer A: 10 mM HEPES, pH 8.0, 10 mM $MgCl_2$, 5 mM mercaptoethanol, 0.1 mM

EDTA. Buffer B: 20 mM HEPES, pH 8.0, 20 mM NH_4Cl, 5 mM mercaptoethanol, 0.1 mM EDTA. Buffer C: buffer B plus 10% glycerol. Buffer D: buffer B but at pH 6.8. Buffer E: buffer C but at pH 6.8. Buffer F: buffer C but at pH 7.8. Buffer G: buffer C except with 100 mM NH_4Cl and 1 mM PMSF. Buffer H: buffer G minus PMSF. Buffer LB: 50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromphenol blue. Binding buffer: 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 5 mM imidazole. Elution buffer: binding buffer with the imidazole concentration raised to 1.0 M.

Pseudouridine formation assay

Reactions contained 50 mM HEPES, pH 7.5, 100 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 100–800 nM [5-^{3}H]uracil-containing $tRNA^{Val}$ transcript, 5 mM DTT, 400 units/mL RNasin, and enzyme. Before addition of DTT, RNasin, or enzyme, samples were placed at 58 °C for 5 min and then at 23 °C for 10 min. Incubation was at 37 °C for various times. Reactions were stopped by addition of 95-μL aliquots to 1.0 mL of 12% Norit A in 0.1 N HCl. Samples were mixed and allowed to stand at room temperature for 5 min, then centrifuged to remove the charcoal; the supernatant was passed through an Acrodisc filter assembly (0.2 μm; catalog #4192, Gelman Sciences). Usually a 0.5-mL sample was counted. One unit of activity is that amount of enzyme catalyzing the release of 1 pmol of ^{3}H to the supernatant in 30 min at 37 °C.

RNA transcripts

Transcripts of the $tRNA^{Val}$ and $tRNA^{Phe}$ genes were prepared from plasmids pVal119 (the gift of J. Horowitz, Iowa State University) and p67CF23 (the gift of O. Uhlenbeck, University of Colorado), respectively. Plasmids were linearized in a reaction mixture containing 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/mL acetylated bovine serum albumin, 0.5 mg/mL plasmid, and 670 units/mL *Bst* N1 for 4 h at 60 °C. Transcription was performed in 40 mM HEPES, pH 7.8, 20 mM $MgCl_2$, 40 mM NaCl, 4 mM spermidine, 10 mM DTT, 5 mM each of ATP, CTP, UTP, GTP, 2 units/mL inorganic pyrophosphatase, 1,000 units/mL RNasin, 33 nM linearized plasmid, 5,000 units/mL T7 RNA polymerase, and 300 μCi/mL of [5-^{3}H]UTP at 37 °C for 7–10 h. The rRNA transcript of full-length 16S RNA (1–1542) was prepared by linearization of pWK1 (Krzyzosiak et al., 1988) and transcription as described above, except with 29 nM plasmid and 400 μCi/mL of [^{3}H]UTP. For synthesis of fragments 1–526 and 1–678 by run-off transcription, pWK1 was linearized with *Sst* II and *Eco* RI, respectively. 23S RNA transcripts were prepared by linearization of pCW1 as previously described (Weitzmann et al., 1990) and transcription as described above for pWK1 except with 22 nM plasmid. Fragment 1–847 was prepared similarly, except that linearization of the plasmid template was with *Eco* RI. All RNA samples were purified by phenol extraction, ethanol precipitation, and gel filtration except for 16S RNA (1–526), which was additionally purified by gradient centrifugation (Weitzmann et al., 1993).

Purification of the tRNA Ψ55 synthase

One hundred grams of *E. coli* MRE600 frozen cell paste (Grain Processing Corp.), harvested in mid-log phase and washed, was thawed at 4 °C with 20 mL of buffer A. After removal of the liquid by centrifugation, the cells were suspended in 250 mL of buffer A plus 10% glycerol and disrupted by sonication. Ribosomes and cell debris were removed by centrifugation at 35,000 rpm for 3 h in a Spinco Ti45 rotor. To the supernatant (S200) was added 1/7 volume of 20% streptomycin sulfate adjusted to pH 7.5, and the mixture was stirred at 4 °C for 30 min. The streptomycin supernatant (272 mL) was recovered by centrifugation and precipitated with ammonium sulfate (152 g). The mixture was adjusted to pH 7–7.5 with NH_4OH and stirred overnight at 4 °C. The precipitate was collected by centrifugation, and the pellet was dissolved in 30 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 × 90-cm column of DEAE Sepharose CL6B equilibrated with buffer C. Elution was with a linear gradient of 0.96 mM/mL NH_4Cl in buffer C. Synthase activity eluted between 250 and 300 mM NH_4Cl. Pooled fractions were dialyzed against buffer D plus 20% polyethylene glycol 6000 to concentrate and remove salt. There was only a 3% loss of activity due to concentration and dialysis. The sample (27 mL) was then loaded on an 8-mL Mono S column equilibrated in buffer E and eluted at 1 mL/min with a linear gradient of 5.56 mM NaCl/mL in buffer E from 0 to 0.5 M, and then at 3 mM/mL from 0.5 to 1 M. The enzyme eluted at 0.58 M NaCl, with a recovery in the pooled fractions of 40% of the input activity. Pooled samples were concentrated and changed into buffer E by filtration through a Centricell 20 (Polysciences, Inc.) membrane with <1% loss of activity. The concentrated sample was run on a second MonoS column as above, but in buffer F and at 0.5 mL/min. Enzyme eluted at 0.28 M NaCl, with a recovery in the pooled fractions of 49% of the input. After Centricell concentration as above, with 98% recovery of activity, the enzyme in buffer F was applied to a 1-mL MonoQ column and eluted at 0.5 mL/min with a gradient of 2.5 mM NaCl/mL in buffer F. The enzyme eluted as a sharp peak (see Fig. 1) at 0.11 M NaCl, with a recovery in the fractions pooled of 48% of input. The pool was concentrated and placed into buffer F by Centricell filtration. Glycerol was added to 50% final concentration and the enzyme stored at −20 °C. The overall recovery of activity based on the pooled fractions from the DEAE column was 9%.

Cloning and overexpression of the tRNA Ψ55 synthase gene

The P35 ORF was amplified and prepared for insertion into pET-15b by PCR. The N-terminal primer extended from −13 to +22, where the A of the initiating AUG is +1, with changes at −1 and −2 to create an *Nde* I site adjacent to the initiating AUG. The C-terminal primer, in the reverse orientation, extended from +927 to +961, where the last sense nucleotide is 942, and contained mismatches at 946, 947, 948, 950, and 951 in order to create a *Bam* HI site. Primers were removed by membrane filtration (Amicon Microcon 100) and the amplified product purified by agarose gel electrophoresis. The pET vector was digested with *Nde* I and *Bam* HI, and without isolation, dephosphorylated with shrimp phosphatase. The trimmed vector was purified by gel electrophoresis before use. The PCR product was digested with *Nde* I and *Bam* HI and directly incubated in a ligation mixture containing

50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 25 μg/mL nuclease-free bovine serum albumin, 10 mM DTT, 1 mM ATP, 500 units/mL T4 DNA ligase, 2 μg/mL vector, and 0.35 or 2 μg/mL insert for 20 h at 16 °C. Transformation of Novablue cells was done by standard methods and yielded two clones with the correct insert in the pET vector out of 20 tested. Plasmids of the two clones were transferred into BL21/DE3 cells.

For overexpression, the transformed BL21/DE3 cells were grown in M9ZB (Studier et al., 1990) at 37 °C to an A_{600} of 0.6. IPTG (1 mM) was added and cells were grown for 3.5 h at 37 °C. Cells were recovered and were quick-frozen on dry ice in aliquots. For analysis of the whole cell contents, one aliquot was thawed in 1/10 the original culture volume of buffer LB, heated to 100 °C for 5 min, and then chilled. Other aliquots were disrupted by sonication in 1/8 or 1/27 the original culture volume of buffer G, and centrifuged at 15,000 × *g* to obtain the S15 supernatant fraction. The pellet was dissolved in 1/24 the original volume of 6 M urea, 0.5 M NH_4Cl, 20 mM HEPES, pH 7.5.

Affinity purification of the synthase

Typically, the S15 supernatant from a 300-mL cell culture whose cells had been sonicated in 1/27 the original volume of buffer G was adjusted to contain 5 mM imidazole and applied to a 2.5-mL column of His-Bind resin. Conditions of preparation and operation of the column were as described in the pET system manual (4th edition, Novagen, Inc.). Despite the presence of 5 mM mercaptoethanol and 0.1 mM EDTA in the 10–12-mL sample applied to the column, the His-Bind column was able to selectively remove the tagged protein from the S15 supernatant. Upon addition of elution buffer, the tagged protein was released. The A_{280}-containing fractions were pooled and dialyzed against buffer H for 4 h before addition of glycerol to 50% and storage at −20 °C. Recovery of input enzymatic activity was approximately 70%.

Polyacrylamide gel electrophoresis

SDS gels were 12% and contained 0.375 M Tris-HCl, pH 8.8, and 0.1% SDS. The 5% stacking gel contained 0.127 M Tris-HCl, pH 6.8, and 0.1% SDS. Samples were heated at 95 °C for 5 min in buffer LB and then quenched on ice before loading. Gels were stained either with Coomassie blue or by using the silver stain reagent kit and protocol from Bio-Rad Laboratories, Inc.

Protein sequencing

Glycerol was removed from the purified enzyme by dialysis, and the protein was precipitated with 9 volumes of cold acetone at −20 °C overnight and then 1 h at −70 °C. The precipitate was dissolved in buffer LB plus 3.5 M urea and electrophoresed as above. Samples were electroblotted onto a PVDF membrane (Millipore Corp.) following standard procedures (Matsudaira, 1987). N-terminal sequencing was carried out as described previously (Denman et al., 1989). We thank Kurt Hollfelder and Yu-Ching Pan of the Department of Protein Biochemistry, Hoffmann-La Roche, Inc., for the sequencing analysis.

Protein determinations

Protein content was assayed by a modified Bradford procedure (Bio-Rad protein assay, catalog #500-0006) using bovine serum albumin as a standard.

ACKNOWLEDGMENT

This study was supported in part by a grant (MRC-MT-1226) from the Medical Research Council of Canada to B.G.L.

Received December 27, 1994; returned for revision January 30, 1995; revised manuscript received February 3, 1995

REFERENCES

Arps PJ, Marvel CC, Rubin BC, Tolan DA, Penhoet EE, Winkler ME. 1985. Structural features of the *his*T operon of *Escherichia coli* K-12. *Nucleic Acids Res 13*:5297–5315.

Bakin A, Ofengand J. 1993. Four newly located pseudouridylate residues in *Escherichia coli* 23S ribosomal RNA are all at the peptidyltransferase center: Analysis by the application of a new sequencing technique. *Biochemistry 32*:9754–9762.

Chang GW, Roth JR, Ames BN. 1971. Histidine regulation in *Salmonella typhimurium*. VIII. Mutations of the *hisT* gene. *J Bacteriol 108*:410–414.

Chen GFT, Inouye M. 1994. Role of the AGA/AGG codons, the rarest codons in global gene expression in *Escherichia coli*. *Genes & Dev 8*:2641–2652.

Ciampi MS, Arena F, Cortese R, Daniel V. 1977. Biosynthesis of pseudouridine in the in vitro transcribed $tRNA^{Tyr}$ precursor. *FEBS Lett 77*:75–82.

Cohn WE. 1959. 5-Ribosyl uracil, a carbon-carbon ribofuranosyl nucleoside in ribonucleic acids. *Biochim Biophys Acta 32*:569–571.

Cohn WE. 1960. Pseudouridine, a carbon-carbon linked ribonucleoside in ribonucleic acids: Isolation, structure, and chemical characteristics. *J Biol Chem 235*:1488–1498.

Cortese R, Kammen HO, Spengler SJ, Ames BN. 1974. Biosynthesis of pseudouridine in transfer ribonucleic acid. *J Biol Chem 249*:1103–1108.

Davis FF, Allen FW. 1957. Ribonucleic acids from yeast which contain a fifth nucleotide. *J Biol Chem 227*:907–915.

Denman R, Weitzmann C, Cunningham PR, Negre D, Nurse K, Colgan J, Pan YC, Miedel M, Ofengand J. 1989. In vitro assembly of 30S and 70S bacterial ribosomes from 16S RNA containing single base substitutions, insertions, and deletions around the decoding site (C1400). *Biochemistry 28*:1002–1011.

Devereux J, Haeberli P, Smithies O. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res 12*:387–395.

Fox JJ, Shugar D. 1952. Spectrophotometric studies of nucleic acid derivatives and related compounds as a function of pH. *Biochim Biophys Acta 9*:369–384.

Goldwasser E, Heinrikson RL. 1966. The biochemistry of pseudouridine. In: Davidson NJ, Cohn WE, eds. *Progress in nucleic acid research and molecular biology*. New York: Academic Press. pp 399–416.

Green CJ, Kammen HO, Penhoet EE. 1982. Purification and properties of a mammalian tRNA pseudouridine. *J Biol Chem 257*:3045–3052.

Johnson L, Söll D. 1970. In vitro biosynthesis of pseudouridine at the polynucleotide level by an enzyme extract from *Escherichia coli*. *Proc Natl Acad Sci USA 67*:943–950.

Johnston HM, Barnes WM, Chumley FG, Bossi L, Roth J. 1980. Model for regulation of the histidine operon of *Salmonella*. *Proc Natl Acad Sci USA 77*:508–512.

Kammen HO, Marvel CC, Hardy L, Penhoet EE. 1988. Purification, structure, and properties of *Escherichia coli* tRNA pseudouridine synthase I. *J Biol Chem 263*:2255–2263.

Krzyzosiak WJ, Denman R, Cunningham PR, Ofengand J. 1988. An

efficiently mutagenizable recombinant plasmid for in vitro transcription of the *Escherichia coli* 16S RNA gene. *Anal Biochem 175*: 373–385.

Lankat-Buttgereit B, Gross HJ, Krupp G. 1987. Detection of modified nucleosides by rapid RNA sequencing methods. *Nucleic Acids Res 15*:7649.

Matsudaira P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem 262*:10035–10038.

Ofengand J, Schaefer H. 1965. On the ionization constant of 5-ribosyluracil. *Biochemistry 4*:2832–2835.

Patton JR. 1994. Formation of pseudouridine in U5 small nuclear RNA. *Biochemistry 33*:10423–10427.

Patton JR, Jacobson MR, Pederson T. 1994. Pseudouridine formation in U2 small nuclear RNA. *Proc Natl Acad Sci USA 91*:3324–3328.

Peattie DA. 1979. Direct chemical method for sequencing RNA. *Proc Natl Acad Sci USA 76*:1760–1764.

Samuelsson T, Olsson M. 1990. Transfer RNA pseudouridine synthases in *Saccharomyces cerevisiae*. *J Biol Chem 265*:8782–8787.

Sands JF, Regnier P, Cummings HS, Grunberg-Manago M, Hershey JWB. 1988. The existence of two genes between *infB* and *rpsO* in the *Escherichia coli* genome: DNA sequencing and S1 nuclease mapping. *Nucleic Acids Res 16*:10803–10816.

Scannell JP, Crestfield AM, Allen FW. 1959. Methylation studies on various uracil derivatives and on an isomer of uridine isolated from ribonucleic acids. *Biochim Biophys Acta 32*:406–412.

Schaefer KP, Altman S, Söll D. 1973. Nucleotide modification in vitro of the precursor of transfer RNA^{Tyr} of *Escherichia coli*. *Proc Natl Acad Sci USA 70*:3626–3630.

Singer CE, Smith GR, Cortese R, Ames BN. 1972. Mutant tRNA^{His} ineffective in repression and lacking two pseudouridine modifications. *Nature New Biol 238*:72–74.

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol 185*:60–89.

Szweykowska-Kulinska Z, Senger B, Keith G, Fasiolo G, Grosjean H. 1994. Intron-dependent formation of pseudouridines in the anticodon of *Saccharomyces cerevisiae* minor tRNA^{Ile}. *EMBO J 13*: 4636–4644.

Tsui HT, Arps PJ, Connolly DM, Winkler ME. 1991. Absence of *hisT*-mediated tRNA pseudouridylation results in a uracil requirement that interferes with *Escherichia coli* K-12 cell division. *J Bacteriol 173*:7395–7400.

Weitzmann CJ, Cunningham PR, Nurse K, Ofengand J. 1993. Chemical evidence for domain assembly of the *Escherichia coli* 30S ribosome. *FASEB J 7*:177–180.

Weitzmann CJ, Cunningham PR, Ofengand J. 1990. Cloning, in vitro transcription, and biological activity of *Escherichia coli* 23S ribosomal RNA. *Nucleic Acids Res 18*:3515–3520.

Yu CT, Allen FW. 1959. Studies on an isomer of uridine isolated from ribonucleic acids. *Biochim Biophys Acta 32*:393–405.

Zamir A, Holley RW, Marquisee M. 1965. Evidence for the occurrence of a common pentanucleotide sequence in the structures of transfer ribonucleic acids. *J Biol Chem 240*:1267–1273.