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A dual-specificity pseudouridine synthase: An *Escherichia coli* synthase purified and cloned on the basis of its specificity for Ψ746 in 23S RNA is also specific for Ψ32 in $tRNA^{phe}$

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ABSTRACT

An *Escherichia coli* pseudouridine (Ψ) synthase, which forms both Ψ746 in *E. coli* 23S ribosomal RNA and Ψ32 in $tRNA^{Phe}$, has been isolated and cloned. The enzyme contains 219 amino acids and has a calculated MW of 24,432 Da. Amino acid sequence comparison with the three other Ψ synthases that have been cloned to date, two for tRNA and one for 16S RNA, did not reveal any common sequence motifs, despite the catalysis of a common reaction. The gene was cloned behind a $(His)_6$ leader for affinity purification. Upon overexpression, most of the enzyme remained soluble in the cell cytoplasm and could be purified to homogeneity on a Ni^{2+}-containing resin. The enzyme reacted with both full-length 23S RNA or a fragment from residues 1–847, forming 1 mol Ψ/mol RNA at position 746, a normal site for Ψ. The enzyme has no dependence on Mg^{2+}. The same yield was obtained in 1 mM EDTA as in 10 mM Mg^{2+}, and the rate was faster in EDTA than in Mg^{2+}. Full-length 16S RNA or fragments 1–526 or 1–678, as well as $tRNA^{Val}$ transcripts, were not modified in either EDTA or Mg^{2+}. $tRNA^{Phe}$ transcripts, however, were modified with a yield of 1 mol Ψ/mol transcript at a rate in EDTA like that of 23S RNA. Sequencing showed all of the Ψ to be at position 32, a normal site for Ψ in this tRNA. Both 23S rRNA Ψ746 and tRNA Ψ32 occur in single-stranded segments of the same sequence, ΨUGAAAA, closed by a stem. Therefore, this synthase may require for recognition only a short stretch of primary sequence 3′ to the site of pseudouridylation. This is the first example of a dual-specificity modifying enzyme for RNA, that is, one which is specific for a single site in one RNA, and equally site-specific in a second class of RNA. The essentiality of these Ψ residues can now be assessed by disruption of the synthase gene.

Keywords: chemical RNA sequencing; large subunit rRNA; modified nucleosides; overexpression; polyhistidine tag; RNA modifying enzyme

INTRODUCTION

Although the number and variety of modified nucleosides in ribosomal RNA (rRNA) is considerable (Maden, 1990; Limbach et al., 1994), their role in rRNA function is largely unknown. This is due in part to the lack of site-specific localization of the modified nucleosides in their respective RNA sequences. This is particularly true for pseudouridine (5-β-D-ribofuranosyluracil, Ψ), which, until recently, could only be located by labor-intensive conventional sequencing procedures (Maden, 1990). With the development of a rapid primed reverse transcriptase approach (Bakin & Ofengand, 1993), sequencing of Ψ residues is no longer a problem. Using this approach, we have determined the location of the single Ψ in *Escherichia coli* 16S RNA (Bakin et al., 1994a), the nine Ψ in *E. coli* 23S RNA (Bakin & Ofengand, 1993; Bakin et al., 1994b), the 30 Ψ in yeast 28S RNA (Bakin et al., 1994b), the 13 Ψ in yeast 18S RNA (Bakin & Ofengand, 1995), and most, if not all, of the Ψ residues in the large subunit (LSU) RNAs of *Halobacter halobium, Bacillus subtilis, Drosphila melanogaster, Mus musculus,* and *Homo sapiens* (Ofengand et al., 1995).

The main conclusion from these analyses was that Ψ residues are clustered in three discrete areas of LSU RNAs, whether they contain 9 residues, as in *E. coli*,

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or 57 residues, as in *M. musculus*, and that all three of these areas are part of, or near to, the peptidyl transferase center (PTC). Moreover, the clustering paralleled the clustering of those methylated residues for which data are available (Bakin & Ofengand, 1993; Brimacombe et al., 1993; Bakin et al., 1994b). Such clustering indicates a nonrandom selection of U residues to be converted to Ψ, and implies the existence of some specific functional purpose to this localization.

We have previously postulated a functional role for Ψ at the PTC (Lane et al., 1992), and in subsequent reports have discussed this and alternate explanations based on the available data (Bakin & Ofengand, 1993; Bakin et al., 1994b; Lane et al., 1995). We believe that the most useful way to approach the question of function is to choose an organism with relatively few Ψ residues, such as *E. coli*, and then attempt to block their formation, one by one, by inactivation of the appropriate Ψ synthase. This approach is predicated on the assumption that each Ψ residue will either require its own specific synthase, as has been found so far to be true for the two rRNA synthases known (Wrzesinski et al., 1995; this work), or that one synthase will form Ψ only at adjacent or nearby sites, as has been found for the *truA* (*hisT*) synthase which forms Ψ at positions 38, 39, and 40 in the anticodon stem and loop of tRNA (Kammen et al., 1988).

Therefore, we have embarked on a program to identify and clone 23S RNA Ψ synthases in *E. coli*, to characterize their site of action and other properties, and to carry out gene disruption and analysis of the in vivo effects of the loss of specific Ψ residues. In this report, we describe the first of our efforts, the identification of the enzyme responsible for the formation of Ψ746 (Fig. 1). Unexpectedly, the purified recombinant synthase also formed Ψ32 in $tRNA^{Phe}$. This is the first example of a dual-specificity modifying enzyme.

RESULTS

Purification of the 23S RNA Ψ746 synthase

Previous work had identified the location of 8 Ψ residues plus one modified Ψ in *E. coli* 23S RNA (Bakin & Ofengand, 1993). Because none of the enzymes were known, our initial experiments were designed to detect any Ψ synthase activity capable of forming one or more of these Ψ residues. For simplicity, we chose as initial substrate free 23S RNA made in vitro (Weitzman et al., 1990) with [5-^{3}H]UTP in order to use the ^{3}H release assay of Cortese et al. (1974) as a measure of Ψ activity. This assay is not completely specific for Ψ, because release of ^{3}H from the 5-position of the uridine ring is also a characteristic of m^5U synthesis and possibly also of the formation of other 5-substituted pyrimidines. In practice, at least under our conditions, most of the activity detected was eventually shown to be due to Ψ synthases. Screening of cell extracts with [5-^{3}H]uracil-labeled 23S RNA transcripts showed that ^{3}H release activity was mostly (>70%) in the S200-streptomycin supernatant fraction and eluted as a sharp peak from a DEAE column. The activity could be further separated into two distinct peaks on a MonoS column. Preliminary studies showed that one peak reacted in an enzyme-dependent manner to yield approximately 1 mol ^{3}H released/mol RNA, whereas the other activity released more ^{3}H and was more affected by changes in the Mg^{2+} and salt concentrations in the assay. The former peak was further purified on a MonoQ column (Fig. 2). At this stage, a polypeptide of ca. 30 kDa was identified whose staining intensity paralleled the enzymatic activity. The specific activity of pooled fractions 40–43 was 1.6×10^6 units/mg protein when assayed at 10 mM Mg^{2+} on RNA (1–847).

Site specificity of the enzyme

Unit stoichiometry (0.9–1.3) was observed whether the assays were done in 10 mM EDTA, 2 mM Mg^{2+}, or 10 mM Mg^{2+} (Table 1). These values were all measured at the plateau of reaction. Table 1 also shows that the same stoichiometry with the same lack of dependence on Mg^{2+} was observed when a fragment of a 23S RNA transcript from residues 1 to 847 was used. These results show that the purified synthase reacted at only a single site in the 23S RNA molecule, that the site remained exposed even in the presence of 10 mM Mg^{2+}, that Mg^{2+} was not required by the enzyme, and lastly that the site in question was within the first 847 residues. Sequence analysis for Ψ (Bakin & Ofengand, 1993) in enzyme-treated 23S RNA and RNA (1–847) showed clearly that the synthase formed only Ψ746 in both cases (Fig. 3). In this method, the RNA is reacted with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC), followed by alkaline hydrolysis to remove CMC from G, U, and most other U-derived bases except Ψ. Subsequent reverse transcription results in a stop one base 3′ to the Ψ residue due to attachment of the CMC group to the N-3 of Ψ,

TABLE 1. Effect of Mg^{2+} and EDTA on the yield of ^{3}H release from full-length 23S RNA and the 1–847 fragment.

Substrate	Conditions	Mol ^{3}H/mol RNA[a]	SD[b]
23S RNA	10 mM Mg^{2+}	1.27 (10)	0.29
(1–2,904)	2 mM Mg^{2+}	1.17 (19)	0.07
	10 mM EDTA	0.93 (4)	0.03
23S RNA	10 mM Mg^{2+}	1.14 (3)	0.09
(1-847)	2 mM Mg^{2+}	1.14 (8)	0.11
	1 mM EDTA	1.14 (3)	0.09

[a] Blank has been subtracted. Value in parentheses is the number of determinations.
[b] Standard deviation.

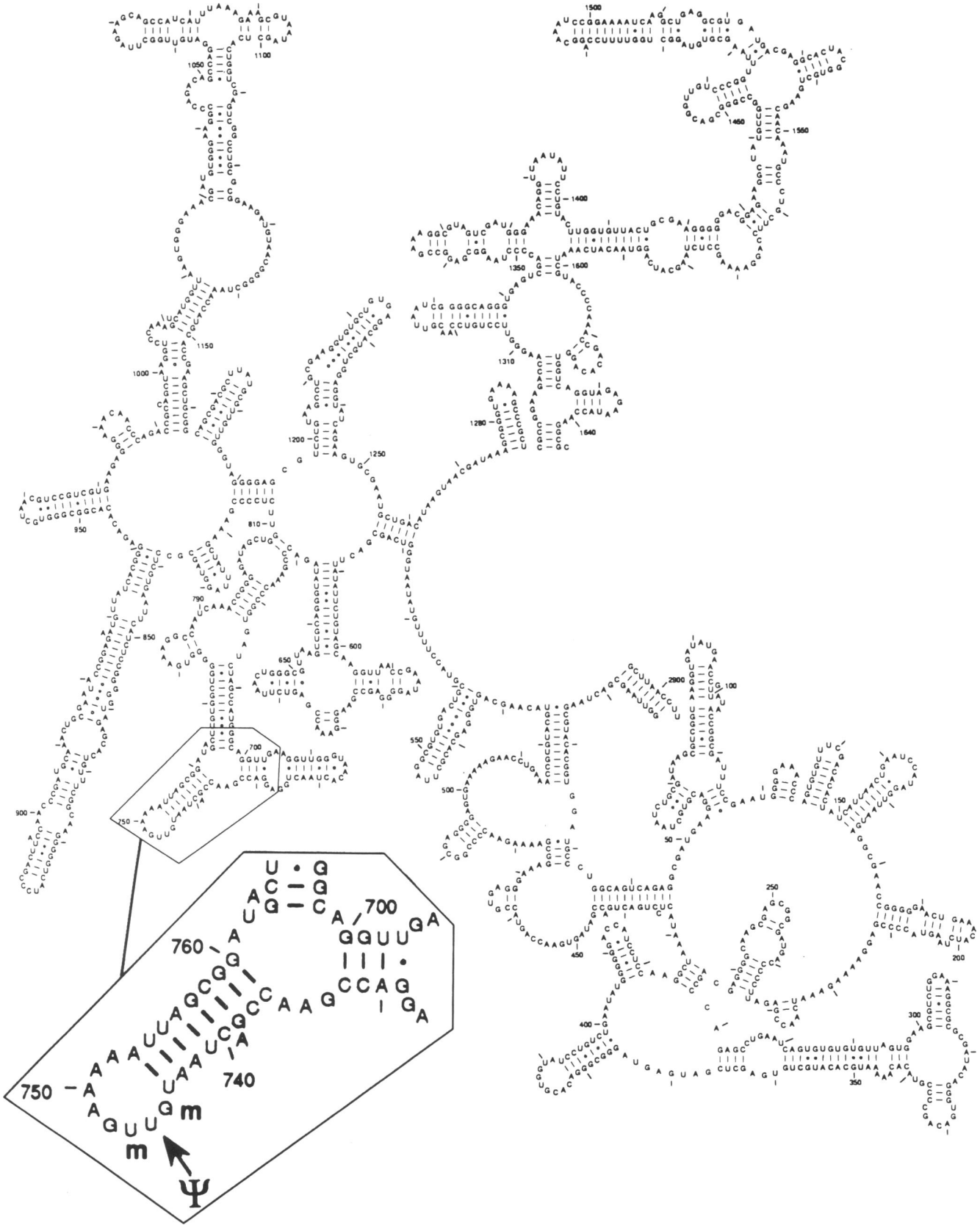

FIGURE 1. Location of Ψ746 in *E. coli* 23S RNA. The 5′-half of the 23S RNA secondary structure (Gutell, 1993) is shown with the segment from residues 696 to 766 in expanded form. mU is m^5U747, mG is m^1G745. Arrow marks the position of Ψ746 (Branlant et al., 1981; Bakin & Ofengand, 1993).

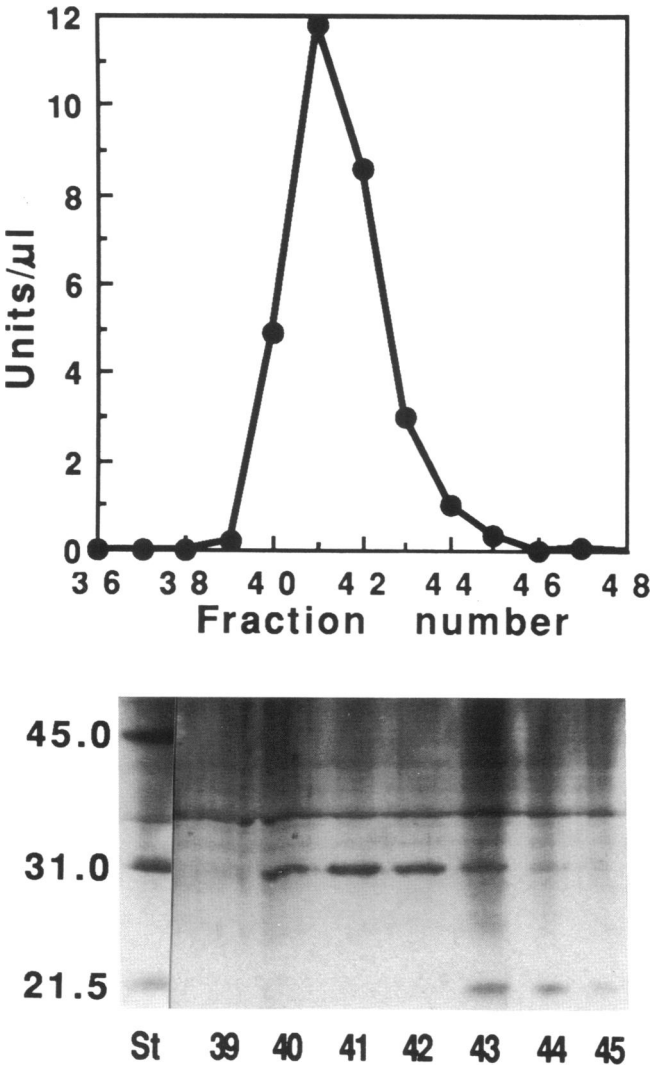

FIGURE 2. Final step of purification of 23S RNA Ψ746 synthase on MonoQ. Upper panel, chromatography of the enzyme on an FPLC MonoQ column was done as described in the Materials and methods. Lower panel, column fractions as indicated were electrophoresed in SDS gels and stained with Coomassie Blue. St, MW standards with values as indicated.

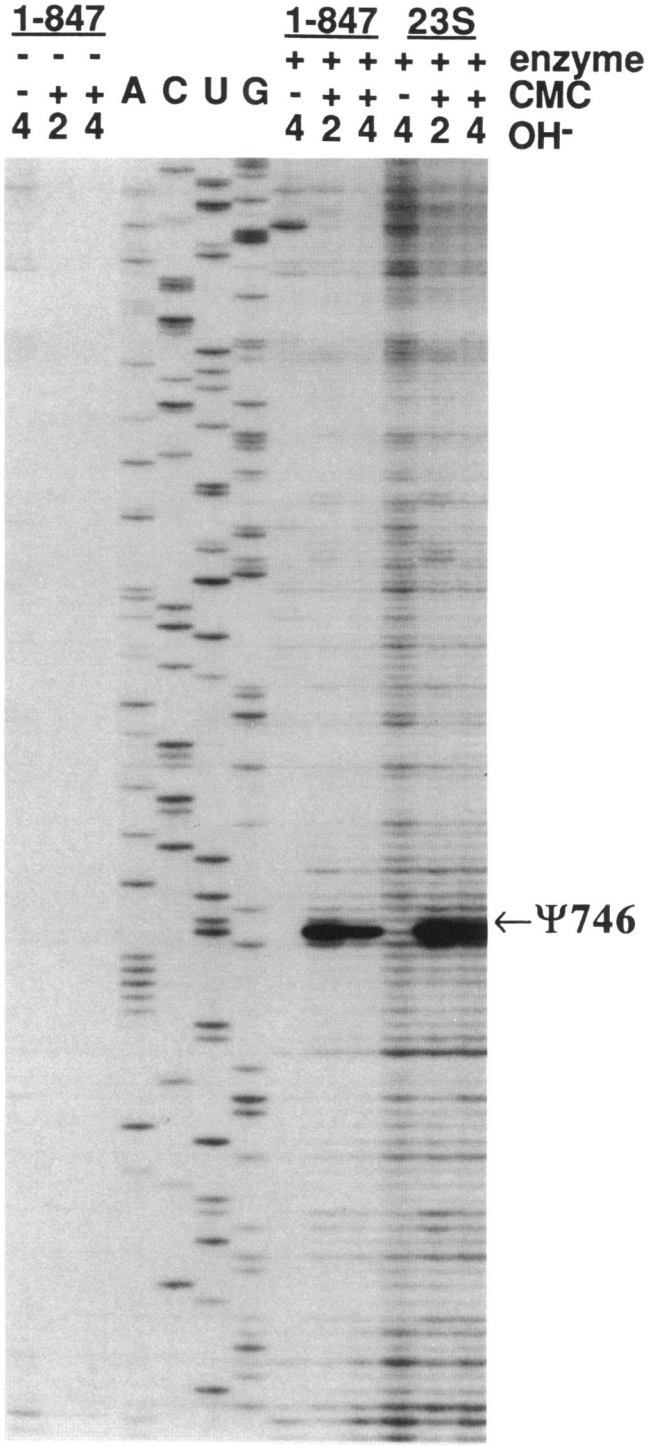

FIGURE 3. Reverse transcription analysis of the site of Ψ formation. In vitro transcript of 23S RNA or the 1–847 fragment that had been incubated with (+) or without (−) enzyme was treated with (+) or without (−) CMC followed by 2 or 4 h incubation at pH 10.4 (OH). The methodology was according to Bakin and Ofengand (1993). A, C, U, G, sequencing lanes using the in vitro transcript. Arrow shows the position of Ψ746.

which blocks base-pairing. Although only residues 647–782 are shown in the figure, residues 437–787 (41%) were screened in this and other experiments. Considering the unit stoichiometry of release as well as the strong bands observed in Figure 3, it is likely that the synthase reacted only with U746. Ψ746 is also the only Ψ found naturally in residues 1–847 (Bakin & Ofengand, 1993).

Identification of the gene

N-terminal amino acid sequencing of the gel-purified protein band shown in Figure 2 yielded the following sequence: emxnYnPpQxpxxv, where the upper case letters denote reliable assignments, lower case letters denote questionable assignments, and x indicates that no assignment could be made. From this unpromising data set containing only three reliable assignments, it was nevertheless possible to select from the GenBank database a putative open reading frame (ORF) whose

TABLE 2. Ψ synthase activity of induced cell extracts with and without the ORF insert.

Source	Units enzyme per mL of extract ($\times 10^{-3}$)[a]	Ratio[b]
pET-15b	5.5	1
Clone 2	235	43
Clone 4	263	48

[a] S15 cell extract was prepared as described in the Materials and methods from induced cells grown at 37 °C and containing the indicated plasmid. The extract was assayed for Ψ formation by the ^{3}H release assay using the RNA (1–847) fragment at 10 mM Mg^{2+}.

[b] Ratio of the activity/mL of clones 2 and 4, respectively, to pET-15b.

deduced N-terminal amino acid sequence, MGMENY NPPQEPWLV . . . , matched the experimental data, including the questionable residues, almost completely. The gene was cloned and, as described below, was shown to indeed code for the 23S Ψ746 synthase. The gene was found in the 0–2.4 min region of the chromosome, accession number D10483, at residues 59,346–60,005 and was in the reverse orientation. There is a normal four-base Shine–Dalgarno sequence separated by six nucleotides from the initiating AUG, and a single UAA termination codon. The gene codes for a 219-amino acid protein with a calculated MW of 24,432 Da.

Cloning of the gene, overexpression, and affinity purification

The ORF was cloned into pET-15b by standard methods. Two clones were selected from the 15 recovered which contained the correct-size insert. Plasmids from these two clones were used to transform BL21/DE3 cells. After induction, S15 extracts from these cells were assayed for ^{3}H release activity. A 40- to 50-fold stimulation was obtained that was dependent on the presence of plasmid containing the insert (Table 2). This figure is probably a minimum value because other ^{3}H-releasing activities would contribute to the pET control value. As shown in Figure 4, this large increase in activity was correlated with the production of a large amount of a protein of about 27 kDa in size. No such band was visible in the induced pET control (data not shown). The majority of the overexpressed protein was found in the S15 supernatant fraction (Fig. 4, lane 1), with a smaller amount of protein in the S15 pellet (lane 2). Most of the protein in the supernatant fraction appeared to be mainly free in the S200 (lane 3). Only smaller amounts were associated with ribosomes (lanes 4, 5). Extracts from cells grown at 30 °C behaved similarly.

The gene was cloned such that its translation product should contain a $(His)_6$ sequence as part of a leader peptide in order to affinity purify the protein on a Ni^{2+}-containing resin column. Ψ synthase activity in such an affinity-purified protein would constitute definitive proof that the gene cloned is the gene for the synthase. Figure 4 (lane P) shows that the Ni^{2+}-column effectively removed all of the contaminating protein from the S15 supernatant fraction, resulting in a single protein band with a monomer MW of 27.0 kDa. Because the 5′-tag increases the MW by 2,179 Da, the expected value for the recombinant protein, 26.6 kDa, based on the MW of 24.4 kDa calculated from the ORF, was in good agreement with what was found.

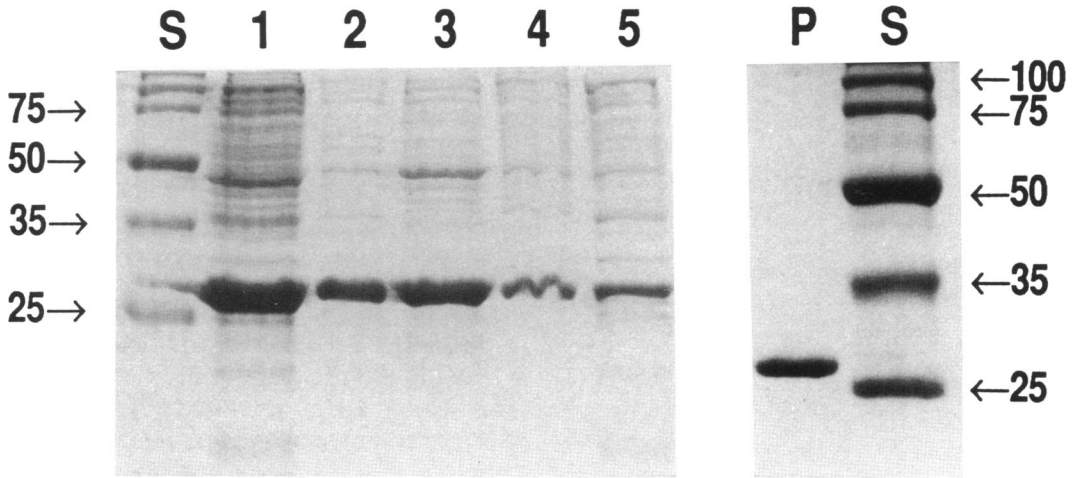

FIGURE 4. Overexpression, cellular localization, and affinity purification of the 23S RNA Ψ746 synthase gene product. Cells grown at 37 °C were broken by sonication and fractionated into an S15 supernatant and a pellet. The supernatant was further fractionated into an S200, a ribosomal 1 M NH_4Cl ribosomal wash (HSW), and ribosomes. Lane 1, S15 supernatant; lane 2, solubilized S15 pellet; lane 3, S200; lane 4, HSW; lane 5, solubilized ribosomes; lane P, 0.3 μg of the affinity-purified recombinant protein. Extracts from approximately equivalent amounts of cells were loaded except for lane P. S, molecular weight standards (Novagen) with values as indicated.

The Ψ synthase enzymatic activity resides in the affinity-purified protein

Treatment of [5-^{3}H]uracil-containing RNA (1-847) with this recombinant protein yielded 1.1 mol ^{3}H released/mol substrate at the plateau of reaction (see also Fig. 6 and Table 3). To verify that Ψ746 was being formed, chemical sequencing was performed (Fig. 5). Although only residues 671-786 are shown in the figure, the sequence from 440 to 796, or 42% of the 1-847 fragment, was screened. Clearly Ψ746 was formed only when the substrate was treated with the affinity-purified protein. In the absence of enzyme treatment, no Ψ was found. Therefore, the affinity-purified protein is, in fact, the Ψ746 synthase and the gene has been identified.

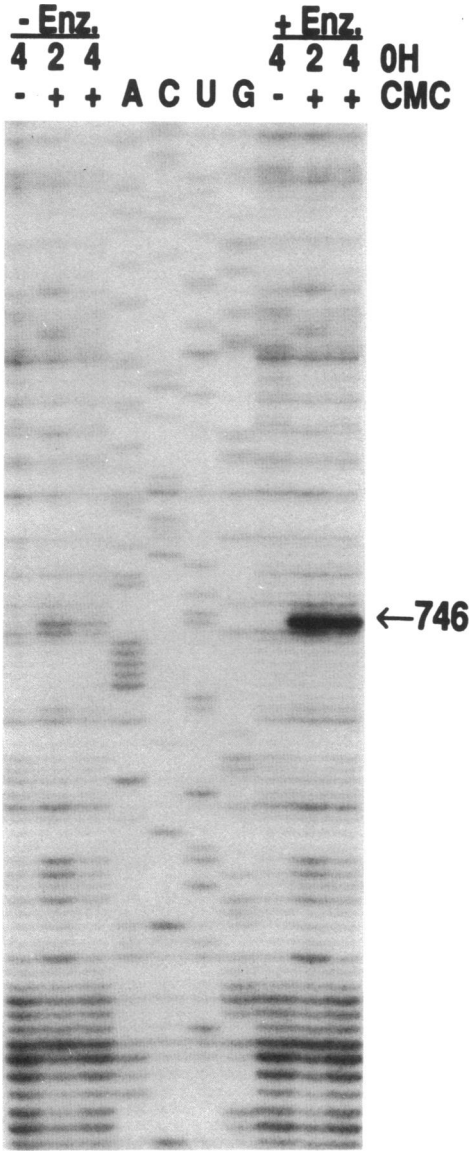

FIGURE 5. Reverse transcription analysis of the site of Ψ formation by the recombinant enzyme. RNA (1-847) that had been reacted with enzyme to a level of 1.1 mol ^{3}H released/mol RNA was treated as in Figure 3. Arrow shows the position of Ψ746.

TABLE 3. Specificity of the recombinant Ψ746 synthase for various RNAs.[a]

RNA transcripts	1 mM EDTA		10 mM Mg^{2+}	
	Rate (pmol/min)	Yield (pmol/pmol)	Rate (pmol/min)	Yield (pmol/pmol)
23S RNA (1-847)	33.12	1.27	7.69	1.19
23S RNA (1-2,904)	7.23	0.97	4.33	–
16S RNA (1-526)	0.04	–	0.02	–
16S RNA (1-678)	<0.01	–	<0.01	–
16S RNA (1-1,542)	0.04	–	<0.01	–
30S subunits	–	–	<0.01	–
$tRNA^{Phe}$	6.21	0.78	0.77	–
$tRNA^{Val}$	0.02	–	0.02	–

[a] ^{3}H release assays were performed as described in the Materials and methods except at 400 nM substrate and 10 mM Mg^{2+} in 100 μL reactions. When 1 mM EDTA was used, Mg^{2+} from the substrate buffer was also present as follows. For RNA (1-847), 0.14 mM Mg^{2+}; RNA (1-2,904), 0.43 mM Mg^{2+}; RNA (1-526), 0.14 mM Mg^{2+}; RNA (1-678), 0.08 mM Mg^{2+}; RNA (1-1,542), 0.12 mM Mg^{2+}; $tRNA^{Phe}$, <0.01 mM Mg^{2+}; $tRNA^{Val}$, 0.02 mM Mg^{2+}. The synthase concentration was 0.45 μg/mL. –, not determined.

The specific activity in EDTA for RNA(1-847) was 22.1×10^6 units/mg, corresponding to a turnover number of 2.77×10^{-2} mol/min/mol of enzyme. For comparison, the equivalent turnover number for the tRNA Ψ55 synthase is 1.05×10^{-2}, or only a threefold difference. Because both enzymes carry out the same reaction on a single-stranded loop of RNA that is closed by a stem, it is reasonable that the turnover numbers are similar. The specific activity of the isolated enzyme (Fig. 2) was 1.6×10^6 units/mg, which should be compared to a value of 5.1×10^6 for the recombinant enzyme when measured under the same Mg^{2+} conditions. If the purity of the isolated enzyme is estimated at ca. 70% (Fig. 2), the specific activity for the pure enzyme becomes 2.3×10^6, or about half that of the recombinant enzyme. Thus, the recombinant enzyme appears to be at least as active as the isolated material.

Evidence for a dual specificity for the Ψ746 synthase and the effect of Mg^{2+}

The specificity of the synthase was tested with transcripts of 16S RNA, both full-length and fragments, and with two tRNA transcripts available in the laboratory. There was no reaction with either full-length 16S RNA or fragments 1-526 or 1-678, nor with $tRNA^{Val}$, whether at 10 mM Mg^{2+} or 1 mM EDTA. 30S subunits in 10 mM Mg^{2+} were also unreactive. To our surprise, $tRNA^{Phe}$ reacted quite well. In the presence of EDTA, the $tRNA^{Phe}$ transcript reacted at virtually the same rate as full-length 23S RNA (Fig. 6A), and reached about the same yield. The 23S RNA (1-847) fragment

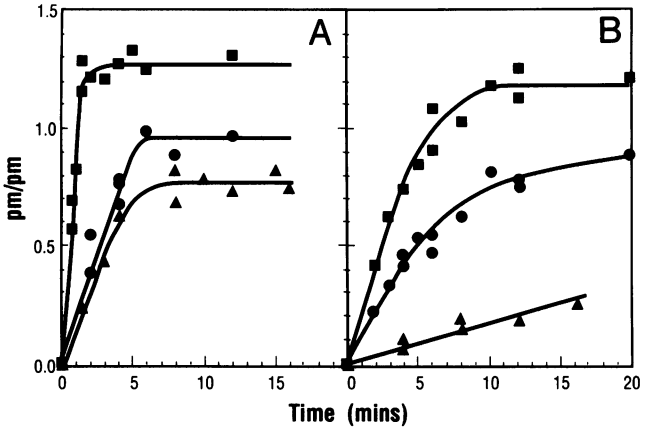

FIGURE 6. Evidence for a dual function for the Ψ746 synthase. [5-^{3}H]uracil-containing in vitro transcripts of ■, RNA (1–847); ●, RNA (1–2,904); and ▲, tRNA^{Phe} were incubated with a fixed amount of affinity-purified recombinant enzyme and Ψ formation assayed as release of ^{3}H as described in the Materials and methods except at 400 nM substrate and (**A**) in the presence of ■, 1 mM EDTA plus 0.14 mM Mg^{2+}; ●, 1 mM EDTA plus 0.43 mM Mg^{2+}; ▲, 1 mM EDTA; or (**B**) 10 mM Mg^{2+}. Pm/pm, pmol ^{3}H released/pmol RNA added to the reaction.

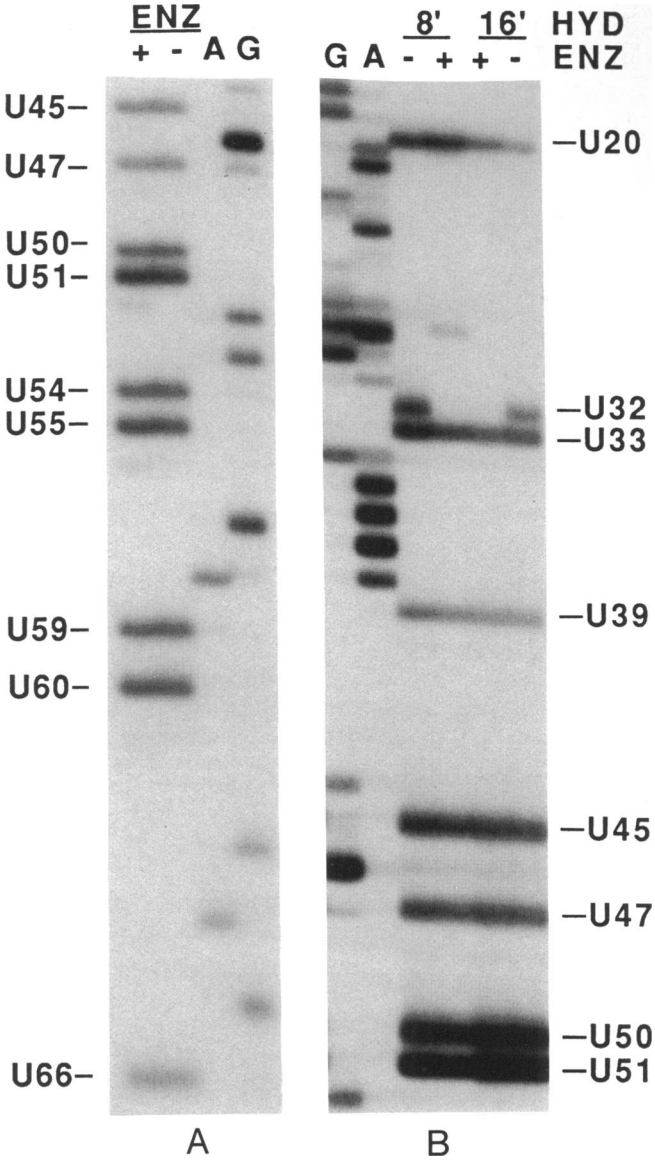

FIGURE 7. Sequence analysis of the site of Ψ formation in tRNA^{Phe} by the recombinant Ψ746 synthase. A [5-^{3}H]uracil-containing in vitro transcript of tRNA^{Phe} reacted with recombinant synthase in 1 mM EDTA to a level of 0.8 mol ^{3}H released/mol of RNA was sequenced. **A:** Chemical sequencing (Peattie, 1979) using 3′-end labeling with [5′-^{32}P]pCp. The reaction of U with hydrazine was for 15 min at 0 °C. Enz +, enzyme-treated RNA; Enz −, control RNA incubated without enzyme. A, G, sequencing lanes using enzyme-treated transcript. The U residues are indicated. **B:** As in A, except that hydrazinolysis (HYD) was performed in 7 M urea at room temperature for the indicated times.

was a better substrate, with a rate almost five times faster, but still yielding about 1 mol ^{3}H released/mol RNA. In the presence of Mg^{2+} (Fig. 6B), all reactions were slowed, and only the one with the RNA (1–847) fragment was rapid enough to reach completion in the allotted time. These results are summarized in Table 3. For two substrates, RNA (1–847) and RNA (1–2,904), rates were also measured in 5 mM EDTA and did not differ from the 1 mM EDTA values. Note that the shift from EDTA to Mg^{2+} affected the three active substrates differently. There was a 4.3-fold reduction for the RNA (1–847) fragment, an 8.1-fold reduction for the tRNA^{Phe} transcript, but only a 1.7-fold drop for the full-length 23S RNA. Mg^{2+}, rather than being a stimulator of this reaction, is inhibitory. The main result, however, is that tRNA^{Phe}, but not tRNA^{Val}, could react with the synthase.

Because the data of Table 3 indicated a single site for Ψ in the tRNA^{Phe} transcript, sequencing analysis was used to determine its location (Fig. 7). Standard chemical sequencing (Fig. 7A) showed the absence of Ψ from U66 (the most 3′-U) to U45. In particular, U55 was not converted to Ψ. In this method, the presence of a band denotes U and its absence indicates Ψ or m^{5}U (Peattie, 1979). The band corresponding to U39 was not visible in this experiment, apparently because this region is too highly structured to allow reaction of U39 under standard conditions. When 7 M urea was included and the hydrazinolysis done at room temperature (Fig. 7B), reaction of U39 was obtained both with and without enzyme treatment. This was also the case for U33 and U20. The only U residue that did not yield a U band after enzyme treatment, but did do so before treatment, was U32. The U16, U12, and U8 bands were also of equal intensity with or without enzyme treatment (data not shown). These results show clearly that U32 was the only U residue converted to Ψ. In particular, U39, which is Ψ in tRNA^{Phe} (Steinberg et al., 1993), was not modified.

Comparison of the sequence surrounding U32 in tRNA^{Phe} with that around U746 in 23S RNA (Fig. 8) revealed the reason for the reactivity of U32 in

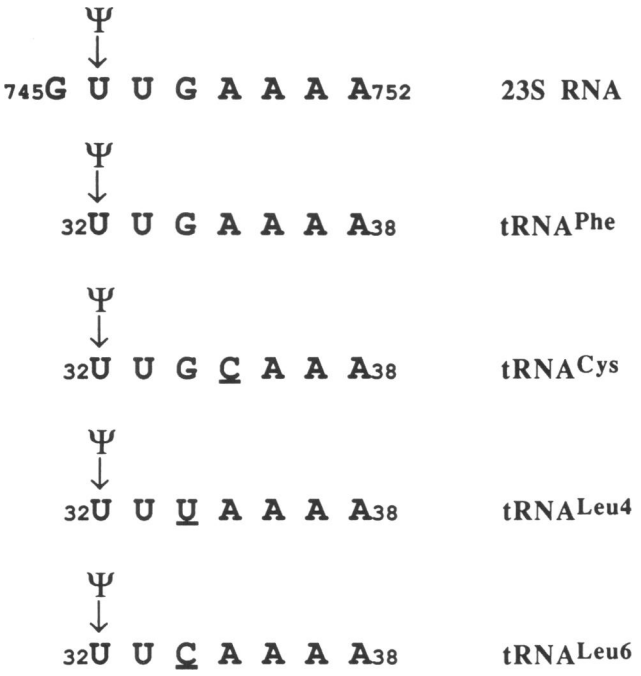

FIGURE 8. Sequence of the single-stranded segments of in vitro transcripts of 23S RNA and $tRNA^{Phe}$ that are recognized by the 23S RNA Ψ746 synthase. Segments of three additional tRNA transcripts are shown that differ by only one base (underlined). tRNA gene sequences are from Komine et al. (1990).

$tRNA^{Phe}$. Both sequences are identical and both are in single-stranded loops. A survey of known *E. coli* tRNA (Steinberg et al., 1993) and tRNA gene sequences (Komine et al., 1990) identified three other tRNAs (shown in Fig. 8) with similar sequences. It will be interesting to determine if the Ψ746 synthase also can form Ψ32 with these transcripts.

Comparison of the amino acid sequence of the synthase with other known proteins

A search of the GenBank database using the TFASTA module of the GCG Sequence Analysis Software suite of programs (Devereux et al., 1984) did not reveal the existence of any other gene product with similarity to the Ψ746 synthase. Moreover, when a comparison of this enzyme (*rluA*) was made with that of the other three cloned Ψ synthases that are known, the *rsuA* synthase that forms Ψ516 in *E. coli* small subunit rRNA (Wrzesinski et al., 1995), the *truA* (also known as the *hisT*) gene product that converts U residues in the anticodon arm of tRNA into Ψ (Arps et al., 1985; Kammen et al., 1988), and *truB*, whose gene product forms Ψ55 in tRNA (Nurse et al., 1995), virtually no amino acid sequence correspondence could be detected (Fig. 9). Despite the common catalytic reaction carried out by these four enzymes, sequence homology at the primary level was not evident.

DISCUSSION

Substrate specificity

In this work, we have described the purification and cloning of an enzyme that site specifically forms Ψ in *E. coli* 23S RNA. This is only the second description of a Ψ synthase for any ribosomal RNA, the first being for 16S RNA (Wrzesinski et al., 1995), and only the fourth example of a cloned Ψ synthase for any RNA, despite the ubiquitious occurrence of Ψ in tRNA, rRNA, and sn(o)RNA. The enzyme formed Ψ at position 746 in 23S RNA. Although this was not rigorously proven by direct analysis of the entire 23S RNA molecule, the fact that approximately unit stoichiometry of ^3H release was obtained both with 23S RNA and with RNA(1–847) (Table 1) argues strongly that all of the Ψ formation occurred in the first 847 nucleotides. Residues 437–796, or 43% of those nucleotides, were directly screened. Moreover, in native 23S RNA, Ψ746 is the only Ψ in the 1–847 sequence (Bakin et al., 1993). Taken together, these facts provide strong evidence that the enzyme described in this work is site-specific with regard to 23S RNA.

Nevertheless, the enzyme also reacted with $tRNA^{Phe}$ transcripts to form Ψ32. The reason for this became apparent when the sequences of the two sites were compared. As shown in Figure 8, the two sequences are identical from the Ψ residue for the next six residues. Apparently, all the enzyme needs is a single-stranded region of an appropriate sequence, although the need for a base paired stem to close the loop cannot be ruled out. In any event, the location of Ψ746 one base removed from the stem, whereas Ψ32 is adjacent to the stem, does not seem to matter. There are no equivalent sequences at any known Ψ site in either 23S RNA (Bakin & Ofengand, 1993; Bakin et al., 1994b) or 16S RNA (Bakin et al., 1994a), in keeping with the evidence that the only reactive site in 23S or 16S RNA is U746 in 23S RNA. The only other known Ψ sites in *E. coli* tRNAs that bear any sequence similarity are Ψ32 in $tRNA^{Cys}$, $tRNA^{Leu4}$, and $tRNA^{Leu6}$, each of which differs by a single base either 2 or 3 residues from Ψ (Fig. 8). Whether the Ψ746 synthase is capable of Ψ32 formation in transcripts of these tRNAs is not known. This is the first example of an RNA modifying enzyme with a dual specificity, that is, one which is completely specific for a single site in one RNA, in this case U746 in 23S RNA, and equally specific for a single site in another class of RNA, here U32 in $tRNA^{Phe}$.

Despite the fact that Ψ746 is located between the modified nucleotides m^1G745 and m^5U747 (see Fig. 1), there is no requirement for either of the modified bases for Ψ formation. The in vitro transcripts of 23S RNA and RNA(1–847) were excellent substrates in the absence of all modified bases. However, the reciprocal need not be true. Formation of m^1G and/or m^5U could require prior Ψ formation.

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      1                                                        50
truB  MSRPRRRGRD INGVLLLDKP QGMSSNDALQ KVKRIYNANR AGHTGALDPL
rsuA  .......... ..... .... .......... .......... ..........
truA  .......... .......... .......... .......... .........M
rluA  .......... .......... .......... .......... .........M

      51                                                       100
truB  ATGMLPICLG EATKFSQYLL DSDKRYRVIA RLGQRTDTSD ADGQIVEERP
rsuA  .......... ..MRLDKFIA QQLGVSRAIA GREIRGNRVT VDGEIVRNAA
truA  SDQQQPPVYK IALGIEYDGS KYYGWQRQNE VRSVQEKLEK ALSQVANEPI
rluA  GMENYNPPQE PWLVILYQDD HIMVVNKPSG LLSVPGRLEE HKDSV.....

      101                                                      150
truB  VTFSAEQLAA ALDTFRGDIE QIPSMYSALK YQGKKLYEYA RQGIEVPREA
rsuA  FKLLPEH.DV AYDGNPLAQQ HGPRYFMLNK PQG...YVCS TDDPDHP...
truA  TVFCAGRTDA GVHGTGQVVH FETTALRKDA AWTLGVNANL PGDIAVRWVK
rluA  ....MTRIQR DYPQAESVHR LDMAT..... .......... SGVIVVALTK

      151                                                      200
truB  RPITVYELL. ....FIRHEG NELELEIHCS KGTYIRTIID DLGEKLGCGA
rsuA  ...TVLYFLD EPVAWKLHAA GRLDID...T TGLVLMTDDG QWSHRITSPR
truA  TVPDDFHARF SATARRYRYI ....IYNHRL RPAVLSKGVT HFYEPLDAE.
rluA  AAERELKRQF REREPKKQYV AR..VWGHPS PAEGLVD... ...LPLICD.

      201                                                      250
truB  HVIYLRRLAV SKYPVERMVT LEHLRELVEQ AEQQDIPAAE LLD...P...
rsuA  HHCEKTYLVT LESPVAD.DT AEQFAKGVQL HNEKDLTKPA VLEVITP...
truA  ......RMHR AAQ...CLLG ENDFTSFRAV QCQSRTPWRN VMHINVTRHG
rluA  ......WPNR PKQKVCYETG KPAQTEYEVV EYAADNTARV VLKPITGRSH

      251                                                      300
truB  ..LLMPMDSP ASDYPVVNLP LTSSVYFKNG NPVRTSGAPL EGLVRVTEGE
rsuA  ..TQVRLTIS EGRYHQVKRM FAAV....GN HVVELHRERI GGITLDADLA
truA  PYVVVDIKAN AFVHHMVRNI VGSLMEVGAH NQPESWIAEL LAAKDRTLAA
rluA  QLRVHML... ALGHPILGDR FYASPEARAM APRLLLHAEM LTITHPAYGN

      301                                  334
truB  NGKFIGMGEI DDEGRVAPRR LVVEYPA...  ....
rsuA  PGEYRPLTEE EIASVV....  ..........  ....
truA  A.TAKAEGLY LVAVDYPDRY DLPKPPMGPL  FLAD
rluA  SMTFKAPADF ..........  ..........  ....
```

FIGURE 9. Sequence comparison of the 23S RNA Ψ746 synthase with all other known Ψ synthases. Sequences of the tRNA Ψ55 synthase (*truB*), the anticodon arm Ψ synthase (*truA*), the 16S Ψ516 synthase (*rsuA*), and this enzyme (*rluA*) were compared using the PILEUP module of the GCG Sequence Analysis Software programs (Devereux et al., 1984). Bold letters show identity or high similarity. C residues are double underlined. Numbers at the left and right margins are amino acid sequence numbers for the *truB* protein.

Gene and protein sequence

The gene was identified as part of a long sequenced region near the origin of the chromosome. We propose the name *rluA* for this gene (*r*ibosomal *l*arge subunit pseudo*U* formation or *U* modification). Comparison of this sequence to the three other Ψ synthase sequences known did not reveal any conserved primary sequence elements, despite the fact that all four enzymes should have the same catalytic center. This might be because the unique nucleotide sequence that is recognized by the enzyme is adjacent to the U to be modified, so that the common amino acid elements of the catalysis site in the enzyme are obscured by the unique amino acid residues needed for recognition. A search of GenBank for analogs of this enzyme in other species was unsuccessful.

Cysteine residues are known to be important for the *truA* (*his T*) Ψ synthase of tRNA (Kammen et al., 1988), and this could be a general feature of Ψ synthases.

Mechanistically, a role for the SH group can be readily envisaged. All four of the known enzymes contain 2–3 cysteine residues, but a comparison of their location in the primary sequence (Fig. 9) did not reveal any common features that might suggest a functional role.

Importance of the enzyme

The significance of this enzyme for *E. coli* depends on the role of Ψ746 in ribosome structure and/or function. Although the region is in the 5'-half of the molecule, it is believed to be part of, or at least physically near, the PTC (Bakin & Ofengand, 1993; Bakin et al., 1994b) and therefore could be envisaged to play a role in peptide bond formation due to the special properties of Ψ (Lane et al., 1992, 1995; Bakin & Ofengand, 1993; Bakin et al., 1994b). However, the position is not one which is universally modified. Analyses of *Halobacter halobium*, *Bacillus subtilis*, *Drosphila melanogaster*, *Mus musculus*, and *Homo sapiens* large subunit ribosomal RNAs did not detect Ψ at this position (Ofengand et al., 1995). Nevertheless, Ψ746 may still be important or even essential for *E. coli*. Gene disruption studies are in progress that should answer the question.

Another aspect is the function of this enzyme in formation of Ψ32 in $tRNA^{Phe}$, and potentially also in $tRNA^{Cys}$, $tRNA^{Leu4}$, and $tRNA^{Leu6}$. These are the only tRNAs in *E. coli* known to contain Ψ32, and all three have sequences that suggest they could be modified by the Ψ746 synthase. It is not clear whether this synthase is the only one capable of Ψ32 formation, or whether there is another, tRNA-specific, synthase in the cell. Moreover, nothing is known about the essentiality of Ψ32 for either tRNA processing/biosynthesis or tRNA function. Disruption of the Ψ746 synthase gene should answer these questions also.

One need also consider that the "real" function of this enzyme may be Ψ32 formation, and that Ψ746 formation is a harmless adventitious event. If gene disruption has no effect, this question becomes trivial. If, however, gene disruption is deleterious and results in loss of Ψ32 for the tRNAs in question, as well as the loss of Ψ746, determination of which Ψ is responsible may be difficult.

MATERIALS AND METHODS

Materials

[5-^{3}H]UTP and [α-^{32}P]dATP were from Amersham. RNasin was from Promega. Restriction enzymes and T4 RNA ligase were from New England Biolabs. T7 RNA polymerase was from Ambion, Inc. Plasmid pET-15b, the BL21/DE3 and Novablue strains of *E. coli*, and His-Bind resin were obtained from Novagen, Inc. T4 DNA ligase and shrimp phosphatase were from U.S. Biochemical. Norit A washed with HCl was obtained from Sigma. Deoxyoligonucleotide primers were prepared as described previously (Bakin & Ofengand, 1993). DEAE Sepharose CL6B, MonoS, and MonoQ FPLC columns were from Pharmacia.

Buffers

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, 10% glycerol. Buffer C: buffer B, but at pH 7.8. Buffer D: 20 mM Tris, pH 8.0, 20 mM NH_4Cl, 5 mM $MgCl_2$, 5 mM mercaptoethanol, 0.1 mM EDTA, 10% glycerol. Buffer E: 20 mM Tris, pH 8.0, 20 mM NH_4Cl, 6 M urea. Buffer LB: 50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.1% bromphenol blue. Binding buffer: 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 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, 2 mM or as indicated $Mg(OAc)_2$, 80–100 nM or, as indicated, [5-^{3}H]uracil-containing full-length 23S RNA or its (1–847) fragment, 5 mM DTT, 400 units/mL RNasin, and enzyme. Mixtures were preincubated for 5 min at 37 °C before adding enzyme. Incubation was then continued at 37 °C for various times. Reactions were stopped by addition of 97-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, centrifuged to remove the charcoal, and the supernatant passed through an Acrodisc filter assembly (0.2 μm, cat #4192, Gelman Sciences). In the earlier experiments, a 0.5-mL sample was counted and the value multiplied by 2. Later, the entire supernatant was removed, and the charcoal pellet washed twice with 1.0 mL of 0.1 N HCl, each time passing the supernatant wash through the same filter assembly as a rinse. The washes and supernatant were combined and counted. Two washes were sufficient to elute 97% of the recoverable ^{3}H. Both methods gave essentially the same results, but the latter was more reproducible. One unit of activity is defined as that amount of enzyme catalyzing the release of 1 pmol ^{3}H to the supernatant in 30 min at 37 °C.

RNA transcripts

The rRNA transcript of full-length 23S RNA (1–2,904) was prepared by linearization of pCW1 (Weitzmann et al., 1990) and transcription 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, 22 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. For synthesis of fragment 1–847 by run-off transcription, pCW1 was linearized with *Eco*R I and transcribed as described. Transcripts of 16S RNA and fragments and of $tRNA^{Val}$ and $tRNA^{Phe}$ were prepared as described previously (Nurse et al., 1995; Wrzesinski et al., 1995). All RNA samples were purified by phenol extraction, ethanol precipitation, and gel filtration.

Purification of the 23SΨ746 synthase

One hundred grams of *E. coli* MRE600 frozen cell paste (Grain Processing Corp.) harvested in mid-log phase and washed, were thawed at 4 °C with 30 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 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 (270 mL) was recovered by centrifugation and precipitated with ammonium sulfate (151 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, the pellet dissolved in 30 mL of buffer B, and dialyzed against the same buffer to remove ammonium sulfate.

The dialyzed sample was loaded at 0.15 mL/min on a 1.5 × 90 cm column of DEAE Sepharose CL6B equilibrated with buffer C and eluted with a linear gradient of 1 mM/mL NH_4Cl in buffer C. Synthase activity eluted at 280 mM NH_4Cl with a recovery of 60% of the input. Peak fractions were pooled and concentrated by dialysis against buffer C plus 20% polyethylene glycol 6000 with no loss of activity. The sample (13.4 mL) was then loaded on an 8-mL MonoS column equilibrated in buffer C and eluted at 1 mL/min with a linear gradient of 1.9 mM/mL in NaCl in buffer C. The enzyme eluted at 210 mM NaCl with a recovery of 35% of the input activity. Pooled samples were dialysed against buffer C, applied to a 1 mL column of MonoQ and eluted at 0.5 mL/min with a gradient of 9.3 mM/mL NaCl in buffer C. The enzyme eluted as a sharp peak (see Fig. 2) at 110 mM NaCl in buffer C with recovery of 60% of the input activity. The pooled fractions were concentrated and placed into buffer C by Centricell filtration. Glycerol was added to 50% final concentration and the samples stored at −20 °C.

Cloning and overexpression of the 23SΨ746 synthase gene

The candidate ORF was amplified and prepared for insertion into pET-15b by PCR. The N-terminal primer extended from −21 to +19 where the A of the initiating AUG is +1, with changes at −1, −3, −4, and −5, to create an *Xho* I site adjacent to the initiating AUG. The C-terminal primer, in the reverse orientation, extended from +671 to +710, where the last sense nucleotide is 657, and contained mismatches at 694, 695, 696, and 697 in order to create a *Bam*H I site. Primers were removed by membrane filtration (Amicon Microcon 100) and the amplified product digested by *Xho* I and *Bam*H I enzymes. The pET vector was digested with *Xho* I and *Bam*H I, dephosphorylated with shrimp phosphatase, purified by gel electrophoresis, and incubated with the gene insert 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, 2.2 μg/mL vector, and 12 μg/mL insert for 20 h at 16 °C. Transformation of Novablue cells was done by standard methods and yielded 15 clones of 25 tested with the correct insert in the pET vector. Plasmids from two clones were transferred into BL21/DE3 cells.

For overexpression, the transformed BL21/DE3 cells were grown in M9ZB (Studier et al., 1990) at 30 °C or 37 °C to an A_{600} of 0.6. IPTG (1 mM) was added and cells grown at 30 °C or 37 °C for 4 h. Cells were disrupted by sonication in 0.15 times the original culture volume (OCV) of buffer D plus 1 mM phenylmethylsulfonylfluoride, and centrifuged at 15,000 × *g* to obtain the S15 supernatant and pellet fractions. The pellet was dissolved in 0.04 OCV of buffer E. The S15 supernatant was centrifuged at 45,000 rpm for 3 h in a Spinco Ti60 rotor to obtain the S200 supernatant and the ribosomal pellet. The top 3/4 of the supernatant was taken as the S200 extract. The ribosomal pellet was suspended in 0.025 OCV of 20 mM Hepes, pH 8.0, 1 M NH_4Cl, incubated at 0 °C for 2 h, and then centrifuged at 200,000 × *g* for 14 h. The supernatant was taken as the ribosomal high salt wash (HSW), and the ribosomal pellet was dissolved in 0.04 OCV buffer E.

Affinity purification of the synthase

The S15 supernatant was dialysed versus binding buffer immediately before application 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. Upon addition of elution buffer, the tagged protein was released. The A_{280}-containing fractions were pooled and dialyzed versus 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, plus imidazole at 0.5 M (1 h), 0.25 M (overnight), 0 M (1 h), and finally versus 20 mM Tris-HCl, pH 7.9, 0.35 M NaCl for 1 h. Under this regimen, the protein stayed soluble. At lower concentrations of salt, or at higher protein concentrations, the protein tends to precipitate. The protein solution was diluted with an equal volume of glycerol and stored at −20 °C at a concentration of 30 μg/mL.

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, 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 Biorad Laboratories, Inc.

Protein sequencing

Glycerol was removed from the purified enzyme by dialysis and the protein precipitated with nine 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).

Protein determinations

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

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