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![Fig. 1. Immunoprecipitation analysis of the reaction between purified human erythrocyte PP-ribose-P synthetase and rabbit serum. Center well contained 3.5 μl of 5000-fold purified normal PP-ribose-P synthetase (460 μg/ml). Numbered wells contained the following: wells 1 and 6, serum from immunized rabbits; wells 2 and 5, serum from unimmunized rabbits; wells 3 and 4, IgG fractions from unimmunized and immunized rabbits, respectively. Double diffusion was done for 24 hr at 4°. Single precipitin bands are noted only where outer wells contained immunoglobulin from rabbits immunized with the purified enzyme.


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Fig. 3. Peroxidase test on 129 morulae (magnification: X800). (a) Preimmunization serum 1:800; (b) antiserum against F9, 1:800.
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Fig. 1. Immunoprecipitation analysis of the reaction between purified human erythrocyte PP-ribose-P synthetase and rabbit serum. Center well contained 25 μl of 5000-fold purified normal PP-ribose-P synthetase (460 μg/ml). Numbered wells contained the following: wells 1 and 6, serum from immunized rabbits; wells 2 and 5, serum from unimmunized rabbits; wells 3 and 4, IgG fractions from unimmunized and immunized rabbits, respectively. Double diffusion was done for 24 hr at 4°. Single precipitin bands are noted only where outer wells contained immunoglobulin from rabbits immunized with the purified enzyme.


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Nucleotide Modification In Vitro of the Precursor of Transfer RNA\textsuperscript{Tyr} of Escherichia coli

(ribothymidine, pseudouridine biosynthesis/cleaved precursor substrate)

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Kline Biology Tower, Yale University, New Haven, Connecticut 06520
Communicated by J. G. Gall, August 20, 1973

ABSTRACT Certain nucleotides in precursor RNA of tRNA\textsuperscript{Tyr} of Escherichia coli were modified in vitro with a preparation of partially purified E. coli enzyme containing ribothymidine- and pseudouridine-forming activity. The only nucleotides modified in vitro are the same as those found modified in mature tRNA. The best substrate for these modifying enzymes is the RNase P cleavage product of the tRNA precursor, which contains the mature tRNA sequence. Of the two pseudouridines found in mature tRNA, one (in the T\textsuperscript{C}C sequence) can be formed in intact precursors. The other (in the anticodon stem) can only be formed in the cleaved precursor RNA. The presence of modified nucleotides in the precursor RNA does not enhance its rate of cleavage by RNase P.

Among cellular RNAs, transfer RNA (tRNA) is unique in its high content of various modified nucleotides (1). Isopentenyladenosine (i\textsuperscript{A}) in tRNA may affect the interaction of tRNA with ribosomes (2, 3), and certain methylated bases in tRNA markedly increase the rate of aminoacylation (4). Some pseudouridines (4) in tRNA play a role in the regulation of the synthesis of amino-acid biosynthetic enzymes (5).

Transcription of tRNA genes is followed by specific cleavage of a larger precursor (6-10). We have used the precursor molecule to a tRNA\textsuperscript{Tyr} of Escherichia coli (7, 8), in which some nucleotide modifications are absent and some present in low amounts, the in vitro RNase P product derived from it (8, 11), and the mature tRNA\textsuperscript{Tyr},\textsuperscript{+} to study in vitro the biosynthesis of ribothymidine, pseudouridine, and (methylthio)isopentenyldenosine. We also investigated the relationship between modification and cleavage of precursor tRNA by RNase P.

MATERIALS AND METHODS

Bacterial Strains. E. coli MRE 600 (RNase P\textsuperscript{-}) was the source of all enzymes. E. coli BP 266 (12) was the host for infection by bacteriophage \$80 psu\textsuperscript{+} A25 (13) used in preparation of tRNA\textsuperscript{Tyr} precursor.

Abbreviations: T, ribothymidine; \$, pseudouridine; i\textsuperscript{A}, N\textsuperscript{1}(\Delta\text{-isopentenyl})adenosine; Gm, 2'-O-methylguanosine; s\textsuperscript{US}, 4-thiouridine; Np, 3'-phosphomonoester of the nucleoside N.
* Present address: Labor fuer Genetik, Universitaet Konstanz, BRD-7750 Konstanz, West Germany.
† To whom reprint requests may be addressed.
‡ Precursor RNA is an RNA molecule 128 nucleotides long containing, internally, the primary sequence of E. coli tRNA\textsuperscript{Tyr} psu\textsuperscript{+} mutant A25 (8). Cleaved precursor RNA is the RNA molecule 87 nucleotides long derived from precursor by cleavage with RNase P. The 5'-end of the product of this reaction begins with the tRNA\textsuperscript{Tyr} psu\textsuperscript{+} mutant A25 sequence. Mature tRNA is tRNA\textsuperscript{Tyr} from psu\textsuperscript{+} mutant A25.

Bacteriophage Strains. Mutant A25 of bacteriophage \$80 psu\textsuperscript{+} has been described (13). This strain carries two mutations in the structural gene for tyrosine tRNA and was used in the preparation of tRNA\textsuperscript{Tyr} precursor.

Chemicals and Reagents. S-Adenosyl l-[methyl-\textsuperscript{3}H]methionine (specific activity 50 Ci/mol) and carrier-free \textsuperscript{32}P-orthophosphate were obtained commercially. T1 and T2 ribonuclease were obtained from Sankyo Co., Tokyo. Ribonuclease A and deoxyribonuclease (free of ribonuclease) were purchased from Worthington. E. coli isopentenylpyrophosphate: tRNA transferase (EC 2.5.x.x) (20) was a gift of Dr. J. Bartz.

\textsuperscript{32}P-labeled tRNA\textsuperscript{Tyr} psu\textsuperscript{+} and Precursor to tRNA\textsuperscript{Tyr} psu\textsuperscript{+} A25 were prepared as described (11) in 20- or 40-ml batches in shaking culture flasks instead of bubbler tubes, which did not affect the yield. After the precursor was extracted from gels, precipitated, and suspended in water, it was dialyzed extensively against glass-distilled water or buffer [20 mM Tris-HCl pH 8-10 mM MgCl\textsubscript{2}] to remove salts coprecipitating in ethanol. Precursor dialyzed in this manner is much less susceptible to contaminating ribonuclease activity found in preparations of modification enzyme. Mature tRNA\textsuperscript{Tyr} and other RNAs used as mobility markers were extracted from the same gels used to prepare precursor. Unfractionated E. coli tRNA used as carrier was a gift of Dr. B. F. C. Clark.

RNase P Was Prepared from 10-g quantities of frozen E. coli MRE 600 cells and assayed as described (11).

For Preparation of RNase P Cleavage Product, which lacks the first 41 nucleotides from the 5'-end of the precursor molecule, dialyzed precursor was incubated with RNase P purified through the DEAE-Sephadex step, as described (11). If a less pure ribosomal wash fraction was used as the source of RNase P, it was dialyzed before use to prevent simultaneous methylation of the substrate.

Preparation of an Enzyme Fraction Containing Uracl tRNA Transmethylase, Other Transmethylase Activities, and Pseudouridine-Forming Activity. The methods used were based on those developed by Johnson et al. (14). Method A: E. coli MRE 600 was grown in rich medium to stationary phase, harvested, and quickly frozen. 30 g of cells was ground with 60 g of alumina (all operations were done at 4\textdegree). The paste was extracted with 100 ml of 20 mM Tris-HCl (pH 8)-10 mM MgCl\textsubscript{2} 20 mM 2-mercaptoethanol and centrifuged for 20 min at 20,000 × g. The resulting supernatant was centrifuged for 2 hr at 100,000 × g. The high-speed supernatant was made 1% in streptomycin sulfate, stirred for 15 min, and centrifuged for 10 min at 27,000 × g. The resulting supernatant was fractionated by ammonium sulfate precipitation. The 33-
47% saturation precipitate was collected by centrifugation and dissolved in 20 ml of 20 mM Tris·HCl (pH 7.8)–1 mM MgCl₂–20 mM mercaptoethanol–10% (v/v) glycerol. This solution (about 20 ml) was dialyzed overnight against a large volume of column buffer [10 mM Tris·HCl (pH 8.9)–5 mM MgCl₂–1 mM EDTA–10% (v/v) glycerol] and then applied to a DEAE–cellulose column (2 × 30 cm) equilibrated with the same buffer. The column was washed with 200 ml of column buffer and then with the same volume of this buffer containing 0.1 M NaCl. Relatively little protein was washed off during these steps. The bulk of the protein, together with the uracil tRNA transmethylase, was eluted by washing with 300 ml of column buffer containing 0.3 M NaCl. Fractions of 5 ml were collected every 5 min. The peak of enzymatic activity lagged slightly behind the peak of protein concentration. The peak fractions of enzymatic activity were pooled and subjected to ammonium sulfate precipitation. The 40–60% saturation precipitate was dissolved in 3 ml of column buffer and dialyzed extensively against 1 liter of this buffer. The enzyme was stored at −20°C in 50% glycerol. The uracil·tRNA methylase is stable under these conditions for at least 6 months. This protein fraction (designated modification enzyme) contained various modifying enzyme activities. Maximum specific activity of enzyme preparations obtained by this method was 30 mU/mg. One enzymatic unit transfers 1 amole of methyl group onto unfractionated Mycoplasma sp. Kid tRNA (at 10 A₅₉₀ units/ml of assay solution) per min.

**Method B:** This method is similar to method A but with the following exceptions: (a) After alumina was removed from the cell lysate, RNase-free DNase was added to a final concentration of 1 μg/ml and the mixture was incubated for 30 min at 4°C. After centrifugation for 4 hr at 100,000 × g, the upper three-quarters of the supernatant were taken. The subsequent streptomycin step was omitted and ammonium sulfate fractionation was done as in method A. (b) An additional elution step with column buffer containing 0.2 M NaCl was performed during DEAE–cellulose chromatography. Most protein was eluted in this step and the levels of RNase activity was generally reduced in all fractions. The specific activity of uracil tRNA transmethylase so prepared was 160 mU/mg. Fig. 1 shows the elution profile of the DEAE–cellulose column.

The significant difference between these two methods is that there is little pseudouridine-forming activity in the 0.3 M NaCl eluate from the DEAE–cellulose column of method B preparations.

**Uracil tRNA Transmethylase Activity Was Assayed** according to Johnson et al. (14) with Mycoplasma sp. Kid tRNA (gift of Margaret Edson) as substrate and S-[¹⁴C]adenosylmethionine as methyl donor.

**Nucleotide-Sequence Analysis of Radioactive RNA.** The modified or unmodified radioactive RNAs were examined by two-dimensional chromatography (15, 16).

**Modified Nucleosides in tRNA Were Identified** by two-dimensional thin-layer chromatography of the 3′-mononucleotides produced by ribonuclease T2 hydrolysis of RNA (14, 17). The nucleotide content was determined quantitatively by scraping the areas containing radioactive material from cellulose plates after two-dimensional chromatography and counting the samples in a liquid scintillation counter. Molar yield of Tp, for example, is calculated by summing all radioactivity found in the Tp, Up, and ψp spots and normalizing this number to the theoretically expected number of moles of Up, determined from the primary sequence of the substrate used. The fraction of radioactivity in the Tp spot then gives the molar amount of all Up modified to Tp. Analyses of T2 hydrolysates of intact precursor, tRNA, or cleaved precursor yielded slightly higher values for minor base content than did the corresponding analysis of T1 oligonucleotides containing these bases.

**Modification Reactions with tRNA Precursor, Cleaved Precursor Product, and tRNA** were done in 0.5 ml of incubation buffer [0.1 M Tris·HCl pH 8–5 mM MgCl₂–6 mM 2-mercaptoethanol plus 1.5 × 10⁶ cpm of S-[¹⁴C]adenosylmethionine], containing 0.05–0.75 mg of modification enzyme protein and variable amounts of substrate. The substrate in any reaction mixture was precursor, cleaved precursor, or mature tRNA in radiochemical quantities (10⁴–10⁶ cpm) with added carrier RNA usually present in not more than 0.5 A₅₉₀ unit per reaction (an excess of carrier up to 10 A₅₀₀ units/0.5 ml had no effect on the activity of uracil tRNA transmethylase). The mixture was incubated for 2 hr at 37°C, and the reaction was stopped by addition of an equal volume of redistilled, water-saturated phenol. After it was shaken, the aqueous layer was removed and the phenol phase was extracted once with half the original volume of water. The combined aqueous phases were dialyzed for 5 hr at room temperature against buffer [0.05 M Na-citrate pH 5.3–0.2 M NaCl–0.01 M MgCl₂] and then overnight either against glass-distilled water or incubation buffer without 2-mercaptoethanol. The samples
found next to the anticodon (Uø), in mature tRNA (18). In addition to the above modifications, Uø is found as sU, Gø is found as Gm, and Uø is found as ψ. The extent of modification was estimated by total enzymatic hydrolysis of the RNAs followed by two-dimensional chromatography and autoradiography. The molar yields of various nucleotides are listed in Table 1. The precursor totally lacks Gm and sU, but contains iA, T, and ψ in partial molar yields, whereas mature tRNA contains significantly higher levels of iA, T, and ψ, and traces of sU. Undermodification of the mature tRNA may be due to disruption of some tertiary structure in the tRNA mutants studied (19), or saturation of host modification enzymes by the large amount of RNA substrate produced in phage-infected cells.

The amount of iA, T, and ψ found in precursor varies somewhat in different preparations. While the molar yield of iA is usually low and shows no obvious correlation with that of any other modified nucleotide, we find consistently a ratio of T:ψ close to 0.60, regardless of variation in absolute molar yields of these nucleotides. This result suggests that modification of these two adjacent nucleotides (Uø, Uø) is interrelated in vivo. This relation between modification levels of these two nucleotides need not be preserved in vitro.

In vitro modification

Properties of Modifying Enzyme Preparations. Before use with tRNA^Tyr or its precursor as substrate, the E. coli modification enzyme was characterized with respect to its ability to form T in Mycoplasma sp. Kid tRNA, since this tRNA contains no ribothymidines (14). The formation of T in this tRNA was linear with respect to time for at least 1 hr at 37°C with modification enzyme made by method A or B (Fig. 3A). Because the precursor is a “natural” substrate for and extremely sensitive to certain nucleases, modification enzyme was also tested for its ribonuclease activities. Contaminating

![Fig. 2](image-url)

**FIG. 2.** Primary sequence of E. coli tRNA^Tyr precursor drawn in a hypothetical secondary structure. Nucleotides found modified in the mature tRNA are indicated with their modifications. The uncertainty in the identity of the 3'-terminal nucleotide has been removed and is shown correctly in this diagram (24; J. D. Smith, personal communication).

were lyophilized and then prepared for nucleotide analysis or chromatography.

**RESULTS**

In vivo modification of precursor and tRNA

Precursor extracted from phage-infected cells is partially modified with ψ, and tRNA extracted from these cells is not fully modified. The following bases are modified: (numbering is from the 5’-end of the tRNA sequence) Uø modified to T; Uø modified to ψ; Aø modified to iA (Fig. 2). The position of the ψ modification is the same as reported (8). No ψ is

<table>
<thead>
<tr>
<th>Modified nucleotide</th>
<th>Molar yield</th>
<th>tRNA</th>
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<tbody>
<tr>
<td>iA</td>
<td>0.24 (0.06–0.50)</td>
<td>0.32 (0.04–0.63)</td>
</tr>
<tr>
<td>T</td>
<td>0.37 (0.13–0.70)</td>
<td>1.04 (1.0–1.13)</td>
</tr>
<tr>
<td>ψ</td>
<td>0.67 (0.24–1.07)</td>
<td>1.28 (1.13–1.47)</td>
</tr>
<tr>
<td>Gm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sU</td>
<td>0</td>
<td>Traces</td>
</tr>
</tbody>
</table>

Precursor and tRNA were isolated and analyzed. Molar yields of modified nucleotides were calculated from known primary sequences. The average value of the ratio T:ψ for 15 precursor preparations was 0.57 with a standard deviation of 0.14. Part of the variability in molar yields may be due to variation in specific activity of individual triphosphates during the brief labeling period in different experiments. Ranges are given in parentheses.

* Average of 15 preparations.

* Average of four preparations.

![Fig. 3](image-url)

**FIG. 3.** (A) Characteristics of method B modification enzyme. Transmethylation activity (A). Degradation of precursor (C) and cleaved precursor (O). (B) Characteristics of endogenous nuclease in method A modified enzyme. Susceptibility of precursor (C), cleaved precursor (C), and tRNA^Tyr (A) to endogenous RNase attack was measured. Control incubation with precursor (Δ) showed no degradation when enzyme was omitted.
ribonucleases in modification enzyme prepared by method A appear to be more active on precursor than on mature tRNA or cleaved precursor (Fig. 3B). This result suggests that the ribonuclease activity is similar to that released from ribosomes washed in 0.5 M ammonium chloride (11). When S-100 fraction is prepared in a manner that minimizes ribosomal disruption and contamination (method B), ribonuclease activity is much reduced (Fig. 3A). Despite various levels of ribonuclease activity in different preparations of modification enzyme, the results of in vitro modification experiments were invariant (Table 2). Although modification enzyme prepared by method A contained more nuclease activity than the method B enzyme, we used it in some experiments because the ψ-forming activity was more active in this preparation. To reduce the possibility that ribonuclease reaction end products (oligonucleotides or undialyzed mononucleotides) interfered with our results, the precursor, after incubation with modification enzyme, was purified on polyacrylamide-gel electrophoresis. The nucleotide composition of this in vitro modified, reisolated precursor was compared with that from precursor that had only been dialyzed after modification; no difference was observed.

**Formation of Ribothymidine and Pseudouridine In Vitro.** Three substrates—precursor tRNA, the RNase P cleavage product of the precursor containing the tRNA primary sequence, and mature tRNA—were used for modification in vitro. After incubation with modification enzyme, the RNAs were analyzed. The results revealed in all cases an increase in Tp and ψp, compared to untreated precursor. With precursor as substrate, the level of Tp was raised significantly (Table 2) with any preparation of modification enzyme, but never to more than 75% of Uα. However, with either cleaved precursor or mature tRNA as substrates, complete methylation could be achieved with either method A or method B enzyme. In vitro modification with method B enzyme, which contains very little ψ-forming activity, resulted in complete methylation of Uα, while modification of Uα to ψ was incomplete. This result suggests that in vitro the uracil tRNA trans-methylase and the ψ-forming activity are not under obligatory interrelated control since they are separable and can function independently (Fig. 1).

Modification enzyme prepared by method A can form about two ψ residues per molecule in cleaved precursor but only one ψ in intact precursor (Table 2). In contrast, method B enzyme in combination with the 0.2 M NaCl fraction can form only one ψ, even in the cleaved precursor. Nucleotide analysis of oligonucleotides, obtained by T1 RNase digestion of the modified RNAs, revealed that only ψp was formed by method B modification enzyme when the 0.2 M NaCl fraction was added to it. This result suggests that there is a difference in reactivity of the two uridines in tRNA^Tyr^ that can be modified to ψ. The existence of different enzymatic activities forming ψ at different sites in tRNA has been shown in *S. typhimurium* (5). In our experiments the enzyme responsible for formation of ψα was no longer present in the DEAE-cellulose eluates in method B. Since the precursor contains only ψα while the mature tRNA contains both ψα and ψp, it seemed plausible that the pseudouridine site next to the anticodon (Uα) becomes reactive only after the precursor is cleaved. To investigate this possibility, precursor and cleaved precursor, before and after treatment with modification enzyme (method A), were digested with T1 RNase. The oligonucleotides that should contain ψ (according to the sequence of mature tRNA^Tyr^) were checked for the presence of this modified nucleoside. When intact precursor was analyzed, no ψ was found next to the anticodon (ψα) either before or after incubation with modification enzyme. In contrast, cleaved precursor after incubation with the enzyme, contained ψ both in the anticodon loop (ψα) and adjacent to T (ψρ) but nowhere else. Thus, the enzyme forming ψ next to the anticodon only acts after the precursor has been cleaved at the 5'-end to mature tRNA size. The enzymatic activity converting Uα to ψα, as assayed in an S-100 fraction of method A, decreases markedly within a week of its preparation.

In our experiments the enzyme responsible for formation of ψα was no longer present in the DEAE-cellulose eluates in method B. Since the precursor contains only ψα while the mature tRNA contains both ψα and ψp, it seemed plausible that the pseudouridine site next to the anticodon (Uα) becomes reactive only after the precursor is cleaved. To investigate this possibility, precursor and cleaved precursor, before and after treatment with modification enzyme (method A), were digested with T1 RNase. The oligonucleotides that should contain ψ (according to the sequence of mature tRNA^Tyr^) were checked for the presence of this modified nucleoside. When intact precursor was analyzed, no ψ was found next to the anticodon (ψα) either before or after incubation with modification enzyme. In contrast, cleaved precursor after incubation with the enzyme, contained ψ both in the anticodon loop (ψα) and adjacent to T (ψρ) but nowhere else. Thus, the enzyme forming ψ next to the anticodon only acts after the precursor has been cleaved at the 5'-end to mature tRNA size. The enzymatic activity converting Uα to ψα, as assayed in an S-100 fraction of method A, decreases markedly within a week of its preparation.

**Introduction of Isopentenyladenosine into tRNA^Tyr^ Precursor.** The precursor is already modified in vivo and contains iA in low molar yields (Table 1). In an attempt to isopentenylate intact precursor in vitro, the RNA was incubated with partially purified *E. coli* isopentenyl-1-tRNA transferase and [3H]-isopentenylpyrophosphate. After hydrolysis and chromatography of the reaction mixture, the spot in the chromatogram corresponding to the mobility of iA was assayed for both [3H] and radioactivity. From the specific activities we calculated that a maximum of 0.03 mol of additional iA per substrate molecule was formed. In addition to iAp, the spot isolated from the thin-layer chromatogram contains the 2-methylthio derivative of iAp (Agris, P. F., Schaefer, K. & Armstrong, D., unpublished). The low yield of the isopentenylation of precursor in vitro, the low amounts of this nucleotide isolated from preparations of precursor in vivo, and the high level of this modification in mature tRNA suggest that cleaved pre-

### Table 2. In vitro modification of precursor tRNA^Tyr^ A25, cleaved precursor, and tRNA^Tyr^ A25

<table>
<thead>
<tr>
<th>Modification of precursor</th>
<th>Cleaved Precursor</th>
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<tbody>
<tr>
<td>Tp</td>
<td>0.2 → 0.44^a 0.9 → 1.03^a 0.2 → 1.04^b</td>
</tr>
<tr>
<td>ψp</td>
<td>0.35 → 0.56 1.3 → 1.81 0.35 → 1.9</td>
</tr>
<tr>
<td>Tp</td>
<td>0.70 → 0.77^b</td>
</tr>
<tr>
<td>ψp</td>
<td>0.80 → 0.85 0.8 → 0.95</td>
</tr>
</tbody>
</table>

^a Molar yields of modified nucleotides determined in *in vivo* modified RNAs (i.e., before *in vitro* incubation with modification enzyme).

^b Molar yields determined after *in vitro* incubation with modification enzyme.
levels of T and modifying uridine those nucleotides completely used substrates untreated RNase substrate and products. The action of RNase P on precursor containing various levels of modified nucleotides. We anticipated that if rare nucleotides facilitated this cleavage step in tRNA biogenesis it would be observable as a rate effect in vitro.

Part of a preparation of precursor was incubated with modification enzyme. Nucleotide analysis showed that the levels of T and ψ in the treated substrate had increased about 20%, from initial values of 0.69 and 0.8, respectively. Both the untreated and treated precursor were then exposed to RNase P. The rate of reaction, which was linear for about 40 min at 37°C, was measured by quantitation of the isolated substrate and products. The data (not shown) indicated that treated substrate is attacked at a rate about 65% of that of untreated substrate. This result, which was reproduced with different precursor preparations, shows that the velocity of the RNase P reaction must be several-fold greater with totally unmodified than with the modified precursor. Both substrates used in our experiments are fully susceptible to RNase P, since in longer incubation both are cleaved completely by this enzyme.

**DISCUSSION**

We have shown that intact precursor tRNA^Tyr and its RNase P cleavage product, containing the tRNA primary sequence, can serve as substrates for enzymatic activities capable of modifying uridine to ribothymidine or pseudouridine and adenine to isopentenyladenosine in vitro. These reactions appear to be as specific in vitro as they are in vivo, since only these nucleotides found modified in the mature tRNA are modified in vitro. We were unable to demonstrate formation of 2′-O-methylguanosine in vitro. Tertiary structure of the RNA might be important in determining enzyme specificity for this modification (19).

Precursor tRNA contains within it the primary sequence of the cleaved precursor and the mature tRNA. Since the rate of reaction of our modifying enzyme preparation with these two substrates is very different, it appears that secondary or tertiary structure is an important factor in determining these rates. In particular, stoichiometric yields of ribothymidine and both pseudouridines are found only with the cleaved precursor as substrate. Like mature tRNA, this RNA is also much more resistant to a contaminating nuclease found in our partially purified preparations of modifying enzyme.

Since cleaved precursor appears to be the best, but not the only, substrate for these nucleotide modifications, we propose that the low levels of modified bases in intact precursor are simply a reflection of the different reaction equilibria of modification enzymes with intact or cleaved precursor as substrates. The equilibria for each substrate must vary for different modifications such that Gm, for example, is found not at all in intact precursor, whereas ψ^δ is found in molar yields sometimes as high as 1.0. Similarly ψ^θ can be produced in vitro only in cleaved precursor, by an enzymatic activity that appears to be quite distinct from the one responsible for the ψ^δ modification. Since high levels of nucleotide modification interfere with the RNase P reaction, we conclude that cleavage of precursor is a very early event in tRNA biogenesis and that the rare nucleotides, as suggested (8), serve no prerequisite role in specific cleavage of precursor tRNA.

Many different functions for modified nucleotides in tRNA have been proposed (2–4). In a mutant of *S. typhimurium* (5, 22), the loss of two pseudouridines in the anticodon region of tRNA^His and tRNA^Leu led to derepression of the corresponding aminocacid-synthesizing enzymes. However, no total loss of function of tRNA in protein biosynthesis has been shown with any undermodified tRNA and, in fact, *E. coli* mutants lacking ribothymidine in all tRNA species (23) are viable. It appears, therefore, that the absence of any one of the modified nucleotides in tRNA may only marginally reduce the capacity of tRNA to function in translation during protein biosynthesis.

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