Properties of a Transfer RNA Lacking Modified Nucleosides*

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A transfer RNA complete devoid of modified nucleosides was synthesized by in vitro transcription, and some of its properties in aminoacylation and protein synthesis in vitro were studied. For this purpose, a plasmid was constructed which contained a glycine tRNA gene from Mycoplasma mycoides under the promoter of the T7 RNA polymerase, as well as a BstNI restriction site at the 3′-end of the tRNA gene. Cleavage of plasmid DNA with BstNI followed by T7 RNA polymerase transcription in vitro yielded an RNA which was processed with M1 RNA, the catalytic subunit of ribonuclease P, to give a tRNA of mature length.

The tRNA synthesized in this manner can be esterified with glycine in vitro, and the rate of aminoacylation is the same as when using the corresponding fully modified glycine tRNA from M. mycoides.

Furthermore, in protein synthesis in vitro, the tRNA lacking modified nucleosides was essentially as efficient as the corresponding normal glycine tRNA. However, the Escherichia coli extract used in our protein-synthesizing system introduced one modification, pseudouridine, into the in vitro-synthesized tRNA, and it cannot be excluded that this modification has an essential role in protein synthesis.

Many nucleosides in tRNA molecules are post-transcriptionally modified (1). The function of some of these modifications has been elucidated, but the role of the majority remains obscure. The modified nucleosides that are present in the anticodon region of the tRNA molecule are generally assumed to be important in the process of codon reading.

We have been studying the mechanism of codon reading using an in vitro protein-synthesizing system derived from Escherichia coli and have obtained evidence for an unconventional codon reading mechanism where the family box codons (such as, for instance, the four glycine codons, GGU, GGC, GGA, and GGG) may be read without discrimination in the third codon position (2-4). These findings are consistent with data in vivo which show that codons are in certain instances read using this method. In the mitochondria, for instance, all of the family box codons are read using only one tRNA for each box (5-7). We have obtained evidence that also in Mycoplasma mycoides most of the family boxes are read in this way (8, 9). Thus, in this organism, a glycine tRNA with the anticodon UCC is the only tRNA available to read the four glycine codons. We have tested this tRNA in our in vitro protein-synthesizing system and have found that it was almost as efficient in the unconventional reading of the GGU and GGC codons as it was in conventional reading (4). This is the result to be expected for a tRNA that had been specifically designed to read all four codons in a family.

The mycoplasma tRNA\textsuperscript{Gly} contains an unmodified uridine in its wobble position and 6-methyladenosine in position 37. The modification pattern of the corresponding tRNA of E. coli, tRNA\textsuperscript{Gly}, is quite different; the wobble uridine is modified while the adenine at position 37 is not. We have shown that in protein synthesis in vitro this E. coli tRNA has other codon reading properties than the mycoplasma tRNA\textsuperscript{Gly} (4), but it is not clear how the modification pattern in the anticodon loop of these tRNAs influences their reading properties.

In order to investigate the function of modified nucleosides in the tRNA molecule, we have synthesized in vitro a tRNA which is completely devoid of modified nucleosides. Using a method similar to that published recently by Sampson and Uhlenbeck (10), a tRNA\textsuperscript{Gly} was synthesized by T7 RNA polymerase transcription of the M. mycoides tRNA\textsuperscript{Gly} gene. In the present paper, we present some of the properties of this tRNA in aminoacylation and protein synthesis in vitro. The potential of a completely unmodified tRNA as a substrate when studying modification enzymes is demonstrated by the fact that we were able to detect the formation of a modified nucleoside, pseudouridine, when the in vitro-synthesized tRNA\textsuperscript{Gly} was incubated with an extract from E. coli.

MATERIALS AND METHODS

Cloning and Nucleotide Sequence Analysis of the M. mycoides tRNA\textsuperscript{Gly} Gene—DNA from M. mycoides subsp. capri was digested to completion with HindIII and inserted into the HindIII site of the phage vector Charon 28. Plaques were analyzed by hybridization, using purified \textsuperscript{32}P-labeled M. mycoides tRNA\textsuperscript{Gly} as a probe. Insert DNA of one of the hybridizing clones was partially digested with Alul, and one of the resulting fragments, that contained the tRNA\textsuperscript{Gly} gene, was sequenced by the chain termination method of Sanger et al. (11).

In Vitro Mutagenesis—In vitro mutagenesis was performed essentially as described in Ref. 12. Single-stranded DNA was prepared from a M13mp18 clone containing an insert starting from the HindIII site indicated in Fig. 1. A synthetic 24-mer oligonucleotide with the desired mutation (Fig. 1) was purchased from Syn-Tek (Umeå, Sweden) and phosphorylated at its 5′-end using polynucleotide kinase.

Single-stranded DNA (50 ng) together with the oligonucleotide (30 ng) in 10 µl of 10 mM Tris, pH 8.0, 50 mM MgCl\textsubscript{2} was incubated at 100 °C for 3 min and then at 57 °C for 1 h. The mixture was adjusted to a final volume of 35 µl and contained 10 mM Tris, pH 8.0, 50 mM MgCl\textsubscript{2}, 1 mM ATP, 5 mM dithiothreitol, 70 µM concentration each from the four dNTPs, 90 units per ml of Klenow fragment of DNA polymerase I, and 60 units per ml of T4 DNA ligase. Incubation was at 16 °C overnight, and the mixture was used to transform JM109 cells. The resulting clones were analyzed by DNA sequencing.

Purification of M1 RNA—A bacterial strain overproducing M1 RNA, the RNA component of RNase P, was kindly provided by Professor S. Altman (Yale University). Cells were grown overnight in 10 liters of LB medium containing 50 mg of ampicillin/liter. After centrifugation, the cells were suspended in 100 ml of 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA and extracted with an equal volume of phenol. After centrifugation, the aqueous phase was made 1 M with respect to NaCl, and 2 volumes of ethanol were added. The fibrous DNA precipitate was removed and discarded. The remaining precip-
Transfer RNA Lacking Modified Nucleosides

A Charon 28 library of HindIII fragments of M. mycoides was constructed and screened by plaque hybridization using purified tRNA<sup>gly</sup> from M. mycoides as a probe. Several of the most strongly hybridizing clones were found to have a 2.0-kilobase pair insert. The nucleotide sequence of part of this fragment was determined and is shown in Fig. 1. Upstream of the tRNA gene are putative promoter sequences as indicated in the figure. Downstream of the tRNA gene there is a hairpin loop that may be an RNA polymerase terminator site. Immediately upstream of the tRNA promoter, there is a sequence encoding a protein homologous to ribosomal protein S15 of E. coli. This finding will be discussed elsewhere in more detail.

In Vitro Synthesis of tRNA

An outline of the procedure used for the synthesis of tRNA lacking modified nucleosides is shown in Fig. 2. A plasmid was constructed which contained the tRNA gene under the promoter of the T7 RNA polymerase, as well as a BstNI restriction site (CCAGG) at the 3' -end of the tRNA gene. Cleavage of plasmid DNA with this enzyme followed by T7 RNA polymerase transcription in vitro gave a transcript which contained a correct tRNA 3'-CCA terminus. M1 RNA, the catalytic subunit of ribonuclease P, was finally used to produce tRNA of mature length.

Construction of Plasmid and In Vitro Transcription—An M13mp18 clone was constructed with an insert that was identical with the one shown in Fig. 1, except that a 5'-terminal fragment of 147 base pairs was removed by cleavage at the HindIII site indicated in the figure. This clone was subjected to oligonucleotide-directed in vitro mutagenesis to introduce the restriction site of BstNI (CCAGG) at the 3'-end of the tRNA<sup>gly</sup> gene. The alteration from the original sequence "TTT" immediately 3' of the tRNA<sup>gly</sup> gene to "GG" is indicated in Fig. 2.

The M13mp18 insert containing the BstNI restriction site was digested with EcoRI and HindIII, and the 0.7-kilobase insert was purified by agarose gel electrophoresis. The insert was annealed with a synthetic primer and ligated into the HindIII site of the vector M13mp18 to generate a new clone which was designated M13mp18-2. The sequence of the HindIII fragment was determined by the dideoxy chain-termination method (20).

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FIG. 1. The nucleotide sequence of a fragment of M. mycoides tRNA<sup>gly</sup> DNA containing the tRNA<sup>gly</sup> gene. This putative promoter and terminator sites are indicated by the symbols ** and > > > < <, respectively.
Transfer RNA Lacking Modified Nucleosides

The plasmid pTZ/MMGLY contains a BstNI restriction site at the 3'-end of the tRNA gene as well as a T7 RNA polymerase promoter upstream of the tRNA gene. Cleavage of plasmid DNA with BstNI followed by T7 RNA polymerase transcription in vitro gives a transcript which contains a correct tRNA 3'CCA terminus. M1 RNA, the catalytic subunit of ribonuclease P, is finally used to produce tRNA of mature length.

Plasmid pTZ/MMGLY digested with BstNI was used as template during transcription in vitro with T7 RNA polymerase. According to the DNA sequence of pTZ/MMGLY, the resulting RNA should have a size of 219 nucleotides. The results in Fig. 3 show that an RNA of approximately this size was indeed synthesized. The RNA transcript was isolated from unincorporated nucleotides by chromatography on Sephadex G-25.

Processing by M1 RNA Yields tRNA\textsubscript{G\textsc{ly}5}—In order to obtain a tRNA\textsubscript{G\textsc{ly}5} of mature size, the 5'-terminal part of the transcript synthesized as described above was removed by treatment with M1 RNA, the catalytic RNA subunit of RNase P, an endonuclease that attacks the bond between the 5'-terminal nucleotide of the tRNA and the nucleotide 5' of this position (18).

Based on the sequence of the DNA template used in this work, the expected products after M1 RNA processing should be an RNA the size of tRNA\textsubscript{G\textsc{ly}5} (74 nucleosides) as well as a larger fragment (145 nucleosides), representing the sequence 5' of the tRNA moiety in the original transcript. When the transcript obtained as described above was incubated with M1 RNA, the expected fragment of approximately 145 nucleosides was indeed formed together with a fragment corresponding to the tRNA\textsubscript{G\textsc{ly}5} product (Fig. 3). However, as shown in Fig. 3, the putative tRNA is not homogeneous. Instead, three separate species are seen, differing by one nucleotide in size.

In order to estimate the size of these three RNAs, they were labeled at their 3'-end with \textsuperscript{32}P-labeled pCp using RNA ligase and their migration rate during polyacrylamide gel electrophoresis and autoradiographed. The RNA transcript (219 nucleotides), as well as the products after processing (145 and 74 nucleotides, respectively), are indicated by roman numerals I, II, and III, respectively.

![Image](https://example.com/image.png)

**Fig. 2.** Outline of the procedure used for the in vitro synthesis of tRNA lacking modified nucleosides. The plasmid pTZ/MMGLY contains a BstNI restriction site at the 3'-end of the tRNA gene as well as a T7 RNA polymerase promoter upstream of the tRNA gene. Cleavage of plasmid DNA with BstNI followed by T7 RNA polymerase transcription in vitro gives a transcript which contains a correct tRNA 3'CCA terminus. M1 RNA, the catalytic subunit of ribonuclease P, is finally used to produce tRNA of mature length.

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**Fig. 3.** Processing of in vitro-synthesized RNA by M1 RNA. RNA was synthesized by T7 RNA polymerase transcription as described in the text, using \textsuperscript{32}P-labeled ATP as a substrate. RNA, 400 ng, was incubated at 37 °C for 1 h with M1 RNA in 50 mM Tris-HCl, pH 7.6, 10 mM NH\textsubscript{4}Cl, 60 mM MgCl\textsubscript{2}, 5% glycerol. The amounts of M1 RNA added were: none (a), 0.3 ng (b), 3 ng (c), 30 ng (d), and 300 ng (e). Samples were then subjected to denaturing polyacrylamide gel electrophoresis and autoradiographed. The RNA transcript (219 nucleotides), as well as the products after processing (145 and 74 nucleotides, respectively), are indicated by roman numerals I, II, and III, respectively.
Transfer RNA Lacking Modified Nucleosides

produce an RNA with the primary structure of a tRNA but lacking the modified nucleotides normally present in a mature tRNA. The final yield, starting from 600 µg of plasmid and using 25,000 units of T7 RNA polymerase, was approximately 300 µg of tRNA. In the following sections, some properties of this unmodified tRNA will be described.

Aminoacylation of tRNA<sup>Gr</sup> Synthesized in Vitro

Normal tRNA<sup>Gr</sup> from M. mycoides may be efficiently aminoacylated using the glycine:tRNA ligase from E. coli. When the unmodified tRNA<sup>Gr</sup> obtained in vitro as described above was used as a substrate for the E. coli ligase, we found that this tRNA could also be aminoacylated to give approximately 30 pmol of glycine esterified per 1 µg of tRNA. Fig. 6 shows an experiment where the rate of aminoacylation at a tRNA concentration of 60 nM was studied. Normal mycoplasma tRNA<sup>Gr</sup> or the in vitro-synthesized tRNA<sup>Gr</sup> was incubated with <sup>3</sup>H-labeled glycine in an aminoacylation mixture containing E. coli glycine:tRNA ligase. The data show that there is only a very small difference in the rate of aminoacylation between normal tRNA<sup>Gr</sup> and the corresponding unmodified molecule.

Protein Synthesis in Vitro

We have previously described an in vitro protein-synthesizing system which is completely dependent on added glycyl-tRNA (4). We have, furthermore, shown that in this system normal mycoplasma tRNA<sup>Gr</sup> can read all four glycine codons (GGN) with about equal efficiency. The in vitro system, programmed with MS2 RNA, produces mainly MS2 coat protein, and, with mycoplasma glycyl-tRNA as its only source of glycine for protein synthesis, the yield of coat protein is essentially the same as in the presence of all the glycyl-tRNA isoacceptors from E. coli. Using this system, we have tested the translational efficiency of the unmodified tRNA<sup>Gr</sup> synthesized in vitro compared to that of its normal, modified counterpart. In one experiment, the glycine tRNAs were esterified with glycine and then incubated separately in the

Fig. 4. Size estimation of in vitro-synthesized RNAs. tRNA<sup>Gly</sup> from M. mycoides (left lane) as well as the RNA obtained from in vitro transcription and processing as described in the text (right lane) were radioactively labeled by ligating <sup>32</sup>P-labeled pCp to their 3'-ends. The RNA were then subjected to electrophoresis on a 6% polyacrylamide gel under denaturing conditions and finally autoradiographed.

Fig. 5. Determination of the 3'-terminal nucleotide of tRNA<sup>Gly</sup> synthesized in vitro. <sup>32</sup>P-Labeled pCp was ligated to the 3'-end of the RNA obtained from in vitro transcription and processing. The RNA was then subjected to gel electrophoresis, and, after autoradiography, the RNA corresponding to the size of authentic M. mycoides tRNA<sup>Gly</sup> was eluted from the gel. It was digested with RNase T<sub>1</sub> to produce nucleoside 3'-phosphates. The nucleotide being labeled then corresponds to the 3'-terminal nucleoside in the original RNA. The nucleotides were separated by thin layer chromatography and autoradiographed.

5. Furthermore, the 3'-end of the 145-nucleotide 5'-terminal fragment generated by M1 RNA processing was shown to be U (compare Fig. 1). This finding further supports the conclusion that the RNA transcript was processed by an endonucleolytic cut at the expected position.

Thus, T7 RNA polymerase transcription in vitro of a tRNA gene followed by processing with M1 RNA may be used to

Fig. 6. Aminoacylation of tRNA<sup>Gly</sup> lacking modified nucleosides. Glycine tRNAs, 60 nM, were incubated with E. coli glycine:tRNA ligase at 25 °C in 0.1 M sodium cacodylate, pH 7.0, 10 mM MgCl<sub>2</sub>, 1 mM ATP, albumin (0.2 mg/ml), 1 mM dithiothreitol, and 20 µM [<sup>3</sup>H]glycine (1000 mCi/mmol) in a total volume of 0.5 ml. The reaction was terminated by the addition of perchloric acid and the amount of [<sup>3</sup>H]glycyl-tRNA formed was determined. The tRNAs used were tRNA<sup>Gly</sup>-synthesized in vitro (closed circles) or a normal tRNA<sup>Gly</sup> from M. mycoides (open circles).
system in the presence of $^{35}$S-labeled methionine. The products of protein synthesis were separated by polyacrylamide gel electrophoresis and autoradiographed. The results in Fig. 7 show that coat protein was synthesized also when the in vitro-synthesized tRNA$^{Gly}$ was used as the only source of glycine for protein synthesis. Furthermore, the amount of protein produced was approximately the same as when the authentic mycoplasma tRNA$^{Gly}$ was used.

In another experiment, the in vitro-synthesized tRNA was allowed to compete with the normal tRNA$^{Gly}$ during protein synthesis. One of the tRNAs was esterified with $^3$H-labeled glycine and the other with $^{14}$C-labeled glycine, and the tRNAs were then incubated together in equimolar amounts in the protein-synthesizing system. The amount of $^3$H- and $^{14}$C-labeled protein was determined at time intervals, and the results are shown in Fig. 8. The unmodified tRNA$^{Gly}$ was quite efficient in the in vitro protein synthesis; after 5 min of protein synthesis, the incorporation of glycine from this tRNA was approximately 75% of that from the normal tRNA$^{Gly}$.

The results of these experiments demonstrate that the in vitro-synthesized tRNA was essentially as efficient in protein synthesis as the corresponding modified tRNA. However, since a rather crude bacterial extract is used in our in vitro protein-synthesizing system, it was necessary to test whether modifications were introduced into the tRNA during the incubation with the extract. The results of these experiments are described in the following section.

Modification of tRNA by the Protein-synthesizing System

It seemed reasonable to assume that a mycoplasma tRNA$^{Gly}$ that lacks modified nucleosides may serve as a substrate for some of the modifying enzymes of E. coli. Of the tRNA$^{Gly}$ isoacceptors in E. coli (20), the tRNA$^{Gly}$ has the greatest resemblance to the mycoplasma tRNA$^{Gly}$. Pseudouridine, 5-methyluridine, and an unknown derivative of uridine in the wobble position are found in this tRNA, but it contains no modified adenosine, cytidine, or guanosine. One could speculate that these modifications are the most likely to be formed when the in vitro-synthesized tRNA$^{Gly}$ is incubated with an E. coli extract. In order to examine to what extent uridines of the in vitro-synthesized mycoplasma tRNA$^{Gly}$ are modified, we prepared, using the in vitro transcription method described above, a tRNA$^{Gly}$ that was labeled with $^{14}$C]uridine. Since the normal tRNA$^{Gly}$ of M. mycoides contains 6-methyladenosine in position 37, we also wanted to test the possibility that this nucleoside was formed in the E. coli extract. We therefore prepared a tRNA$^{Gly}$ labeled with $^{14}$C]adenosine.

The radioactively labeled tRNAs were incubated in the protein-synthesizing system and purified from the incubation mixture by phenol extraction and fast protein liquid chromatography gel filtration. To analyze the nucleoside content, the resulting preparation of tRNA was enzymatically hydrolyzed with nuclease P1 and bacterial alkaline phosphatase. Nucleosides were finally separated using HPLC and thin layer chromatography.

The results of the HPLC separation are shown in Fig. 9. In the experiment where the RNA was labeled with $^{14}$C]uridine (upper panel), there is only one peak of radioactivity in addition to the one corresponding to uridine. This peak appears in a position corresponding to pseudouridine or possibly dihydrouridine which elutes at approximately the same posi-
Transfer RNA Lacking Modified Nucleosides

FIG. 9. HPLC separation of nucleosides. Transfer RNA, labeled with [\(^{14}\)]C]uridine (upper panel) or [\(^{14}\)]C]adenosine (lower panel) was synthesized in vitro and incubated in the in vitro protein-synthesizing system. The tRNA was purified by phenol extraction and gel filtration on a column of Superose 12. It was hydrolyzed to nucleosides by digestion with nuclease P1 and bacterial alkaline phosphatase, and the hydrolysate was finally subjected to HPLC on a Bondapak C18 column as described under "Materials and Methods."

The amount of radioactivity is 4.9% of that in the uridine peak. This figure corresponds to approximately 1 mol/mol of tRNA since there are in all 22 uridines in the tRNA molecule. It would therefore seem that this modification reaction is very efficient.

In the experiment where the tRNA was labeled with [\(^{14}\)]C] adenosine, the HPLC profile shows two radioactive peaks in addition to adenosine (Fig. 9, lower panel). One is adenosine 5'-phosphate which results from incomplete action of the phosphatase. The other peak is material of unknown identity which is not retained by the column and cannot represent a modified adenosine. These two peaks were present also in the nucleoside analysis of a control sample of [\(^{14}\)]C]adenosine-labeled RNA which was not incubated with the E. coli extract (data not shown). We may therefore conclude that no adenosine residues in the tRNA are modified when this tRNA is incubated in an in vitro protein-synthesizing system.

An analysis by thin layer cellulose chromatography of the [\(^{14}\)]C]uridine-labeled hydrolysate confirmed the presence of pseudouridine and, as in the HPLC analysis, no other modifications of uridine were detected (Fig. 10). From this experiment, formation of dihydrouridine could be excluded since there was no radioactivity in the position corresponding to this nucleoside. A similar analysis of the [\(^{14}\)]C]adenosine-labeled hydrolysate again demonstrated that there were no modified adenosines (data not shown). We may thus conclude that pseudouridine is the only modification to be introduced into the in vitro-synthesized tRNA by the protein-synthesiz-
Transfer RNA Lacking Modified Nucleosides

Fig. 10. Thin layer chromatography of nucleosides. Transfer RNA labeled with [3H]uridine was synthesized in vitro and incubated in the in vitro protein-synthesizing system. The tRNA was purified by phenol extraction and gel filtration, enzymatically digested with pancreatic RNase, and the hydrolysate was subjected to thin layer cellulose chromatography as described under "Materials and Methods." Consequenty, it cannot be excluded that this modified nucleoside has an essential role in protein synthesis in vitro.

Discussion

In vitro transcription with T7 RNA polymerase has become an important method used to obtain large quantities of RNA for various biochemical studies. We have used transcription of a cloned tRNA gene by this enzyme followed by M1 RNA processing of the transcript to produce tRNA molecules in vitro. The tRNA, derived from a mycoplasma tRNA^Gly gene, that we obtained in the present work was shown to have the correct primary structure and to differ from its normal counterpart in that it completely lacked modified nucleosides. We have tested the unmodified tRNA^Gly in aminoacylation and protein synthesis in vitro, and the results show that this tRNA is functional in both these reactions. However, it should be noted that incubation in the protein synthesis system resulted in the introduction of one modification in the tRNA, pseudouridine, and this will be discussed further below.

Aminoacylation—Using the glycine:tRNA ligase from E. coli, the unmodified tRNA^Gly was aminoacylated at the same rate as normal tRNA^Gly from M. mycoides. The normal tRNA has only two modifications, 4-thiouridine (s^4U) in position 8 and 6-methyladenosine (m^6A) in position 37. It has been shown that for some tRNAs s^4U is not essential for the aminoacylation reaction in vitro (21). Furthermore, m^6A is not present in glycine tRNAs from E. coli. It would therefore seem unlikely that these two modifications in the mycoplasma tRNA^Gly are important for the recognition of this tRNA by the E. coli glycine:tRNA ligase. Thus, our finding that the unmodified and the fully modified tRNAs are charged with glycine at the same rate is not unexpected.

Protein Synthesis in Vitro—Normal tRNA^Gly from M. mycoides can sustain protein synthesis in vitro under conditions when it is the only source of glycine for protein synthesis. Furthermore, we have previously shown that it is almost as effective in the unconventional reading of the GGU and GGC codons as in the conventional reading of the GGA codon (4). The E. coli tRNA^Gly isoaceptor on the other hand is not as efficient in unconventional reading. This latter tRNA has a modification pattern in the anticodon loop which is different from the mycoplasma tRNA; the wobble uridine is modified, and the adenosine in position 37 is unmodified. However, it is not clear how the modification pattern in these two tRNAs influences their codon reading properties.

In the present work, we have demonstrated that the tRNA^Gly also, which lacks the modifications normally present in the normal mycoplasma tRNA^Gly, is efficient in protein synthesis. Approximately the same amount of MS2 coat protein is formed by the protein-synthesizing system regardless of whether the in vitro-synthesized tRNA or the normal modified tRNA is used as a source of glycine for protein synthesis. When these two tRNAs were allowed to compete with each other in the protein-synthesizing system, the rate of glycine incorporation as well as the yield obtained with the unmodified tRNA was almost as high as that with fully modified tRNA. However, as already pointed out, this incubation introduced one modification, pseudouridine, into the synthetic tRNA as discussed below.

Work is now in progress to ascertain whether the unmodified tRNA^Gly reads the individual glycine codons in the same manner as the fully modified tRNA. The results of these experiments might, for instance, be informative as to the role of the m^6A in position 37. It has been suggested that this type of modification would prevent too strong binding of the tRNA to the ribosome-mRNA complex (1), but it cannot be excluded that it also influences the reading of the third codon position. The s^4U in position 8 of the normal tRNA^Gly, on the other hand, is probably not essential for protein synthesis since an E. coli mutant lacking this modification is able to grow normally (22).

Modification of tRNA by the Protein-Synthesizing System—When the tRNA synthesized in vitro was incubated in the protein-synthesizing system, one modified nucleoside, pseudouridine, was formed as demonstrated by both HPLC and thin layer chromatography analyses. Since the pseudouridine present in position 55 is the only one found in E. coli glycine tRNAs, it is reasonable to assume that in the synthetic mycoplasma tRNA it is the uridine in this position that is converted to pseudouridine when the tRNA is incubated with the bacterial extract. The amount of pseudouridine formed corresponds to 1 mol/mol of tRNA, i.e. the conversion to pseudouridine is very efficient during the 10-min incubation with the protein-synthesizing system.

Our translational system has obviously not been optimized for the modification of different nucleosides, and such reactions often require different kinds of cofactors. The fact that only pseudouridine was formed in consistent with previous reports that no low molecular weight substrates are required in this reaction. Furthermore, since pseudouridine is present in all tRNAs in E. coli, the isomerization of uridine to pseudouridine might be expected to be a particularly efficient modification reaction.

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Transfer RNA Lacking Modified Nucleosides


