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Codon reading properties of an unmodified transfer RNA

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We have previously shown that the *Mycoplasma mycoides* glycine tRNA (anticodon UCC) effectively reads the codons GGU and GGC in violation of the classic codon reading rules. We have attempted to elucidate what structural elements in this tRNA molecule confer this translational property and in the course of this investigation T7 RNA polymerase transcription of the corresponding gene was used to produce a tRNA devoid of modified nucleosides. Using an in vitro translation system the ability of this tRNA to read the 4 glycine codons (GGU, GGC, GGA and GGG) was tested and it was shown to be as efficient as its normal, fully modified counterpart in the reading of all four codons. This result demonstrates that a tRNA devoid of modified nucleosides is able to efficiently sustain protein synthesis in vitro and, furthermore, that the normal modification pattern of the *Mycoplasma* glycine tRNA is not essential for the ability of this tRNA to read the glycine codons GGU and GGC effectively.

Mycoplasma; tRNA; Codon; Anticodon; Modified nucleoside

1. INTRODUCTION

Using an in vitro translation system we have examined the recognition between the tRNA anticodon and the mRNA codon. We have discovered that certain codons may be read without discrimination between the nucleotides that can occupy the third codon position. For instance, a *Mycoplasma mycoides* glycine tRNA (anticodon UCC) was unexpectedly efficient in its unorthodox reading of the codons GGU and GGC [1]. This tRNA is the only glycine tRNA present in this organism and it appears to have been designed to read the 4 glycine codons with comparable efficiency. This type of codon reading in *Mycoplasma* does not seem to be restricted to the glycine codons. We have made an inventory of the tRNAs and tRNA genes in *Mycoplasma mycoides* that correspond to the family box codons, i.e. the groups of 4 codons where all codons in each group have the two first nucleotides in common and specify the same amino acid. We have shown that for the majority of amino acids corresponding to such family boxes, there is only one tRNA available to read all 4 codons [2–4]. Thus, the situation in this organism resembles that of mitochondria [5–7] and chloroplasts [8].

We have also shown that the 3 different glycine tRNAs from *E. coli* are not as efficient as the *Mycoplasma* glycine tRNA in this type of unconventional reading [1]. For instance, the $tRNA_2^{Gly}$ (anticodon NCC, where N is a modification of U) is not as efficient in its reading of GGU and GGC and the $tRNA_1^{Gly}$ (anticodon CCC) reads GGA, GGU and GGC only with difficulty. This is essentially the result predicted by the wobble rules. In an attempt to investigate what structural features of a glycine tRNA molecule are important for its ability to read the glycine codons in an undiscriminating manner we have studied the properties of tRNAs obtained by mutagenisation in vitro of the corresponding genes. Replacement of the wobble nucleotide in $tRNA_1^{Gly}$ with U so that it now has the same anticodon as the mycoplasma tRNA, results in a tRNA which is still very inefficient in its reading of GGU and GGC [9]. This means that the anticodon UCC is not per se sufficient for undiscriminating reading and that the structural context provided by the tRNA molecule is also important in this respect.

One structural difference between the *Mycoplasma* and *E. coli* glycine tRNAs is in the pattern of modified nucleosides [10]. The *Mycoplasma* tRNA contains only two such nucleosides, 6-methyladenosine in position 37 and 4-thiouridine in position 8 [3]. The glycine tRNAs from *E. coli* have more modifications and the $tRNA_2^{Gly}$ has a modified uridine in its wobble position but adenosine in position 37 is unsubstituted [10]. However, it is not clear how the modification pattern of the different glycine tRNAs influences their codon reading properties. In order to test the importance of modified nucleosides for the translational properties of glycine tRNAs we developed a method to produce a tRNA completely devoid of modified nucleosides by using in vitro transcription with T7 RNA polymerase [11]. The tRNA synthesized in this manner can be efficiently esterified with glycine in vitro. In protein synthesis in vitro programmed with MS2 RNA the tRNA lacking modified nucleosides appeared to be essentially as efficient as the corresponding normal glycine tRNA.

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However, it should be noted that the *E. coli* extract used in our translational system introduced one modification, pseudouridine, in the in vitro-synthesized tRNA [11].

In the present paper we have further investigated the translational properties of the unmodified tRNA and have measured the ability of this tRNA to read the individual glycine codons. To do this we have made use of a recently developed system [9] which enables us to conveniently monitor the reading of the four different glycine codons.

2. MATERIALS AND METHODS

2.1. *Materials*

Radiolabelled chemicals were obtained from Amersham or from New England Nuclear. The *E. coli* strain overproducing T7 RNA polymerase, HMS12/pGP1-5/gGP1-1, was generously provided by Stanley Tabor (Harvard Medical School). The preparation of T7 RNA polymerase [12] and M1RNA [11] has been described.

2.2. *Aminoacylation of tRNA*

Glycine tRNA from *Mycoplasma mycoides* was purified to homogeneity by chromatography on benzoylated DEAE-cellulose as previously described for other tRNAs from this organism [3]. Unmodified tRNA was produced as previously described [11]. Glycyl-tRNA synthetase was prepared from *E. coli* as described in [14] and was used for the aminoacylation of the glycine tRNAs. The normal, fully modified *M. mycoides* glycine tRNA and its unmodified counterpart were esterified with ^{14}C- or ^{3}H-labeled glycine at a specific radioactivity of 114 mCi/mmol and 1.0 Ci/mmol, respectively. The incubation mixture contained 0.1 M sodium cacodylate buffer, pH 7.0, 10 mM $MgCl_2$, 1 mM ATP, 0.2 mg/ml BSA, 1 mM dithiothreitol, 2.0 μM radioactive glycine, 0.45 μM tRNA and glycyl-tRNA synthetase. The total volume was 0.5 ml and incubation was at 25°C for 30 min. The reaction was terminated by the addition of 100 μl 1 M sodium acetate, pH 4.5. The glycyl-tRNAs were finally extracted with phenol, precipitated with ethanol and dissolved in 50 μl 50 mM ammonium acetate, pH 4.5.

2.3. *In vitro protein synthesis*

Protein synthesis was carried out using the in vitro system previously described [1,9]. The incubation mixture contained 40 mM Tris-HCl, pH 7.8, 40 mM KCl, 6.7 mM ATP, 0.2 mM GTP, 56 μM mercaptoethanol, 7.5 mM phosphoenol pyruvate, 11.2 mM magnesium acetate, 16 μg/ml pyruvate kinase, 0.6 mg/ml *E. coli* tRNA, 0.2 mg/ml tetrahydrofolate, 2 mM concentration of each amino acid, an S30 extract of *E. coli* ts48, and finally 1.2 mg per ml of messenger RNA prepared as previously described [9]. For the experiments shown in Fig. 1 the incubation mixture contained in addition [^{35}S]methionine (75 μCi, 1000 Ci/mmol) and 2.0 μM glycyl-tRNA. For experiments in which two tRNAs competed with each other the reaction mixture contained the tRNAs in concentrations of 1.0 μM each. One of them was esterified with [^{14}C]glycine and the other with [^{3}H]glycine. The final volume was 250 μl and incubation was for 15 min at 37°C. RNAse, 20 μg, was added and the mixture was incubated for 30 min at 37°C. Finally 10 μl 5 M NaOH was added and incubation was continued for 30 min at 37°C. The coat-protein fragment produced by the translational system was purified by gel filtration on a Sephadex G25 column and by CM-Sepharose chromatography as described in the legend to Fig. 2.

3. RESULTS AND DISCUSSION

3.1. *Synthesis of unmodified tRNA*

The construction of the plasmid pTZ/MMGLY and the synthesis of tRNA using this plasmid has recently been described [11]. The *Mycoplasma* glycine tRNA gene with a *Bst*NI restriction site at its 3′ end was under the promoter of T7 RNA polymerase. Cleavage of plasmid DNA with *Bst*NI followed by in vitro transcription resulted in a transcript with a correct tRNA 3′-CCA terminus. M1RNA, the catalytic subunit of ribonuclease P, was finally used to produce a tRNA of mature length. The product had the primary structure of *Mycoplasma* glycine tRNA but lacked the modified nucleosides normally present in this tRNA.

3.2. *In vitro protein synthesis.*

For the present investigation we made use of a recently developed in vitro translation system which allowed us to conveniently monitor the reading of the 4 different glycine codons [9]. We used 4 different messenger RNAs that were obtained by transcription with T7 RNA polymerase of cDNA clones of the viral message MS2 RNA that had been mutagenized in vitro. Each RNA contains only one kind of glycine codon in the coat protein cistron. Thus, we have one RNA where GGU is the only glycine codon in this cistron, one that contains only GGC etc. Furthermore, two consecutive stop codons were introduced into the coat protein cistron resulting in a truncated coat protein product. For further details of this translational system see [9]. Our protein-synthesizing system was strictly dependent on added glycyl-tRNA as the S30 extract used in the experiments described below was prepared from an *E. coli* mutant with a temperature-sensitive glycyl-tRNA synthetase [1].

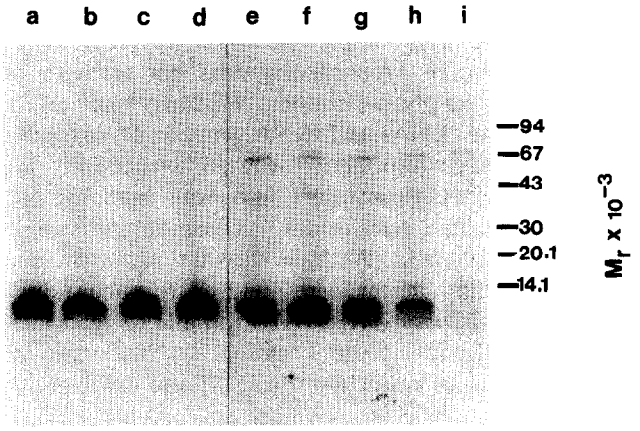

Fig. 1. Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis of products of the in vitro translational system. Protein was synthesized in vitro in the presence of [^{35}S]methionine and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Phast system (Pharmacia LKB, Uppsala, Sweden) with PhastGel Gradient 8–25. In lanes e–h normal, fully modified *Mycoplasma* glycine tRNA esterified with glycine was added to the system and in lanes a–d the corresponding in vitro transcript lacking modified nucleosides were used instead. In lane i no glycyl-tRNA was added. Each of the messenger RNAs used contained only one type of glycine codon, GGG (lanes a and e), GGC (lanes b and f), GGU (lanes c and g), and GGA (lanes d, h, and i), respectively. For further details see sections 2 and 3.

3.3. *The unmodified tRNA effectively reads the individual glycine codons to produce a coat protein fragment of the expected size.*

In vitro protein synthesis was carried out in the presence of [^{35}S]methionine and a tRNA isoacceptor charged with glycine. The glycine carried by this tRNA was the only source of this amino acid for protein synthesis. The products of translation were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and the results are shown in Fig. 1. In an experiment where no glycyl-tRNA was added to the protein synthesizing system only negligable amounts of the coat protein fragment were produced (Fig. 1, lane i). When the normal mycoplasma tRNA charged with glycine was present in the system the coat protein fragment was synthesized regardless of which of the four different MS2 RNAs were used (Fig. 1, lanes e–h). This finding supports our previous conclusion that the mycoplasma tRNA effectively reads all 4 glycine codons [1]. Furthermore, when the tRNA lacking modifications was the only source of glycine in the system the yield of a coat protein fragment of the expected size was essentially the same (Fig. 1, lanes a–d) demonstrating that this tRNA is also able to read all 4 glycine codons.

To compare the reading efficiency of the unmodified glycine tRNA produced in vitro to that of the normal mycoplasma glycine tRNA experiments were performed in which these tRNAs competed with each other for the same codon. One tRNA was esterified with [^{3}H]glycine and the other with [^{14}C]glycine and the tRNAs were incubated together in equimolar amounts in the in vitro protein synthesizing system. The radiolabelled coat protein fragment produced was purified by gel filtration and ion-exchange chromatography (Fig. 2). This procedure effectively removed radioactive polypeptide contaminants as shown by Borén et al. [15]. The peak fractions from the ion-exchange chromatography were pooled and the ^{3}H- and ^{14}C-radioactivity was determined. A relative reading efficiency was obtained by comparing the ratio of ^{3}H to ^{14}C to that of the isotope ratio of the glycyl-tRNAs in the incubation mixture during protein synthesis.

The results shown in Table I demonstrate that for each of the individual glycine codons the reading efficiency of the unmodified glycine tRNA is very similar to its normal counterpart. This suggests that the modified nucleosides normally present in the mycoplasma glycine tRNA, m^{6}A in position 37 and s^{4}U in position 8, are not essential for the ability of this tRNA to read the codons GGU and GGC effectively. As mentioned above, the bacterial extract used in the in vitro protein synthesizing system introduces one modification, pseudouridine, in the tRNA synthesized in vitro and it is therefore not possible to exclude a role of this particular nucleoside. It should be noted, however, that it is present also in the *E. coli* tRNAs and it therefore seems unlikely that it is important for undiscriminating reading.

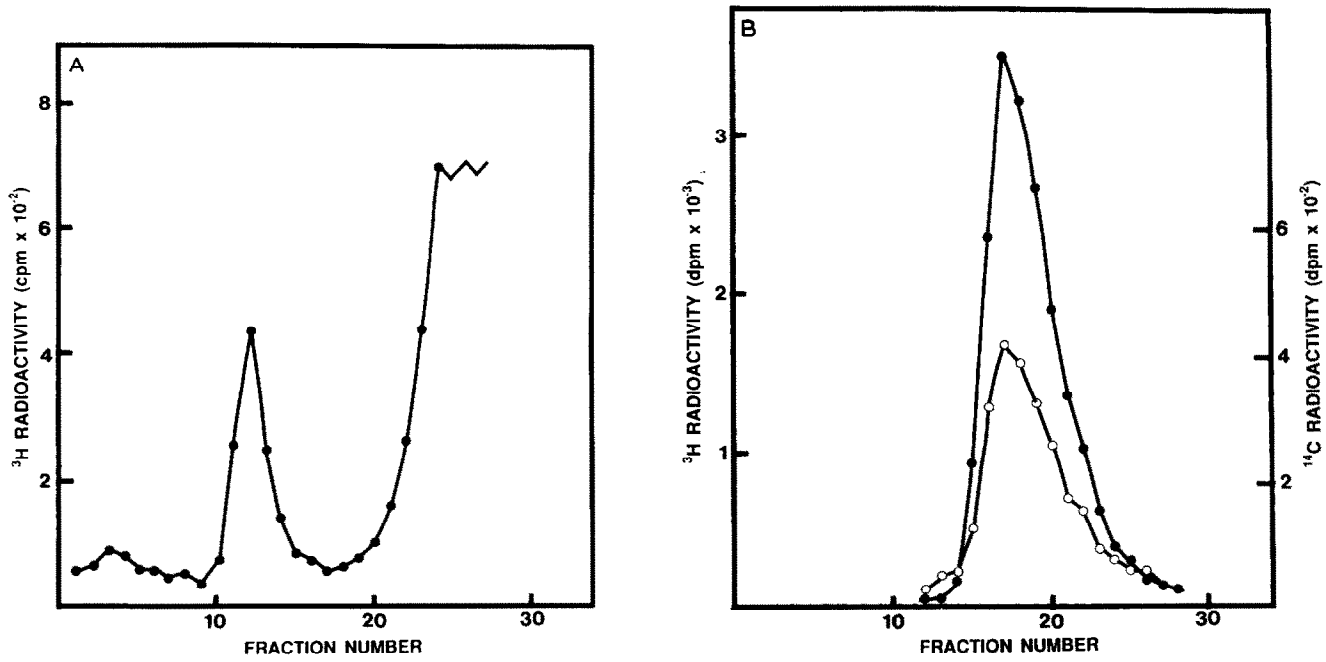

Fig. 2. Purification of the MS2 coat protein fragment obtained by protein synthesis in vitro. (A) The coat protein fragment produced by in vitro translation was first purified by gel filtration on a Sephadex G25 column (1 × 25 cm). Elution was with 8 M urea, 0.1 M MSH, 20 mM MOPS buffer, pH 7.5 (buffer A). Fractions of 0.5 ml were collected and the radioactivity of 50 μl aliquots was determined. Fractions containing the fragment (in this experiment 11–14) were combined. (B) Further purification was achieved by chromatography on a CM-Sepharose column (1.5 × 5 cm). The column was first washed with 10 ml of buffer A and the fragment was then eluted with a linear gradient of NaCl using 10 ml of buffer A in the mixing chamber and 10 ml of 1 M NaCl in buffer A in the reservoir. Fractions of 0.5 ml were collected and the radioactivity of 50 μl aliquots was determined. Filled circles represent ^{3}H and open circles ^{14}C radioactivity.

Table 1

Efficiency of unmodified glycine tRNA in reading the glycine codons

Glycine codon	Relative reading efficiency				
	Experiment no				Mean value ± standard deviation
	1	2	3	4	
GGU	0.6	1.6	0.6	0.9	0.9 ± 0.4
GGC	0.6	1.8	0.6	0.9	1.0 ± 0.6
GGA	1.0	1.4	0.8	1.5	1.2 ± 0.3
GGG	0.3	0.9	0.4	1.0	0.7 ± 0.3

The reading efficiency of unmodified glycine tRNA produced in vitro was compared to that of the normal mycoplasma tRNA by allowing these tRNAs to compete with each other for the same codon. One tRNA was esterified with [^{3}H]glycine and the other with [^{14}C]glycine and the tRNAs were incubated in the in vitro protein synthesizing system. The coat protein fragment produced was purified and the ^{3}H- and ^{14}C-radioactivity was determined. The relative reading efficiency was obtained by comparing the ratio of ^{3}H to ^{14}C to that of the isotope ratio of the glycyl-tRNAs in the incubation mixture during protein synthesis.

As already noted we have previously presented evidence that the normal *Mycoplasma* glycine tRNA reads the codons GGU and GGC with unusual efficiency [1]. Our interpretation of these experiments was based on the assumption that there is only one glycine tRNA in *Mycoplasma mycoides* and all available data support this assumption [3]. Nevertheless, we could not completely exclude the possibility of an additional glycine tRNA in *Mycoplasma* which is present in low concentration in our tRNA preparation. Such a hypothetical tRNA might effectively read GGU and GGC thereby giving the erroneous impression that the major glycine tRNA does not discriminate between the glycine codons. However, this possibility now seems extremely unlikely in view of the present results which show that the tRNA obtained by in vitro transcription, which is definitely homogeneous, has the same unconventional reading properties as the tRNA isolated from *Mycoplasma* cells.

It is interesting to note that a tRNA produced by in vitro transcription lacking modified nucleosides is completely functional during protein synthesis in vitro. It should therefore be possible to use such RNAs for many biochemical studies of tRNA. A technical problem sometimes encountered in studies of tRNA is that such molecules when isolated from cells are difficult to obtain in large amounts and in a highly purified state. However, using T7 RNA polymerase transcription homogeneous RNAs are easily obtained in mg quantities.

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