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The nucleotide in position 32 of the tRNA anticodon loop determines ability of anticodon UCC to discriminate among glycine codons

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ABSTRACT **We have investigated the influence of structures in the tRNA anticodon loop and stem on the ability of the anticodon to discriminate among codons. We had previously shown that anticodon UCC, when placed in the structural context of $tRNA_1^{Gly}$ from *Escherichia coli*, discriminated efficiently between the glycine codons, as required by the wobble rules. Thus, this anticodon read GGA and GGG but did not read GGU and GGC, whereas in mycoplasma $tRNA^{Gly}$, the same anticodon did not discriminate among the glycine codons. We have now determined the reading properties of three constructions based on $tRNA_1^{Gly}$ containing the anticodon UCC in different structural contexts. In one of these constructs, $tRNA_1^{Gly}$-ASL, the anticodon loop and stem are the same as in mycoplasma $tRNA^{Gly}$. The second construct, $tRNA_1^{Gly}$-AS, has an anticodon stem identical with the mycoplasma $tRNA^{Gly}$, whereas in the last construct, $tRNA_1^{Gly}$-C32, the only difference from $tRNA_1^{Gly}$(UCC) is that the uridine in position 32 of the anticodon loop has been replaced by cytidine. These constructs were tested for ability to read glycine codons in an *in vitro* protein-synthesizing system that allowed us to monitor separately the reading of each codon. We found that the anticodon UCC, when present in $tRNA_1^{Gly}$-AS, discriminated among the glycine codons, whereas in the constructs $tRNA_1^{Gly}$-ASL and $tRNA_1^{Gly}$-C32, the same anticodon had lost its ability to discriminate—i.e., it behaved as in mycoplasma $tRNA^{Gly}$. These results strongly suggest that nt 32 of the anticodon loop of $tRNA_1^{Gly}$(UCC) decisively influences the reading properties of the anticodon UCC.**

Analysis of tRNA genes and their gene products in mitochondria (1–3) has revealed that in these organelles each family box (group of four codons that have the same nucleotides in the first two positions and that all denote the same amino acid) is read by only one tRNA, which cannot discriminate among the nucleotides in the third-codon position, as required by the wobble rules (4). A similar situation exists in chloroplasts (5, 6), although in this case only about half of the codon families are read without discrimination. Our own analysis of *Mycoplasma mycoides* indicated that in this microorganism the family boxes, except for the threonine box, are read by only one tRNA each (7–9). The same results have been obtained in *Mycoplasma capricolum* by Andachi *et al.* (10). tRNAs that read without discrimination among nucleotides in the third-codon position, in most cases, have an unsubstituted uridine as their wobble nucleotide.

These results are reminiscent of a phenomenon we had previously observed in a protein-synthesizing *in vitro* system programmed with the phage messenger MS2 RNA. In this system we found that family boxes could be read by a single tRNA with an undiscriminating method we referred to as "reading by two-out-of-three" (11). The capacity for this unconventional type of reading varied among different isoaccepting tRNAs. The $tRNA^{Gly}$(UCC) from *M. mycoides* (12) was almost as efficient in the unconventional reading of codons GGU and GGC as it was in conventional reading, whereas $tRNA_1^{Gly}$(CCC) from *Escherichia coli* can be regarded at the other end of the spectrum in that it discriminates very intensely among glycine codons in our *in vitro* system, as required by the wobble rules (13).

We had shown that the anticodon UCC, when present in the structural context of the $tRNA_1^{Gly}$ molecule, behaved as predicted by the wobble rules—i.e., it read GGA and GGG but did not read GGU and GGC, whereas in the structural context of the mycoplasma $tRNA^{Gly}$, it read without discrimination among the nucleotides in third-codon position, in violation of the wobble restrictions (14). Obviously there must be structural elements outside the anticodon that are important for the ability of a tRNA to discriminate among the nucleotides that occupy the third-codon position.

In the present paper we have investigated the influence of structures in the anticodon loop and stem on the discriminatory ability of anticodon UCC. The results strongly indicate that the nature of nt 32 of the anticodon loop determines whether or not UCC will discriminate among glycine codons.

MATERIALS AND METHODS

Preparation of S30 extract from *E. coli* ts48 and purification and esterification of tRNAs were as described (13). The T7 RNA polymerase was purified, as described by Tabor and Richardson (15). T4 DNA ligase, T4 polynucleotide kinase, and restriction endonucleases were from Boehringer Mannheim. Oligonucleotides were synthesized by using a model 380A DNA synthesizer (Applied Biosystems) or obtained from the Department of Physical Chemistry, Chalmers University of Technology (Göteborg, Sweden). Oligonucleotides from trityl-on syntheses were purified by using the oligonucleotide-purification cartridges from Applied Biosystems. Vector pKK 233-3 was from Pharmacia, and the pTZ vector was from Promega. Expression vector pET-3 and *E. coli* BL21(DE3)pLysS were provided by F. W. Studier (Brookhaven National Laboratory, Upton, NY).

Construction and Cloning of $tRNA_1^{Gly}$ Mutants. $tRNA_1^{Gly}$(UCC) was obtained as described (14). The following three $tRNA_1^{Gly}$-based mutants were constructed, all containing anticodon UCC: $tRNA_1^{Gly}$-ASL (where the anticodon loop and stem are the same as in mycoplasma $tRNA^{Gly}$), $tRNA_1^{Gly}$-AS (where the anticodon stem is identical with $tRNA^{Gly}$ from *M. mycoides*), and $tRNA_1^{Gly}$-C32 (where uridine in position 32 of the anticodon loop has been replaced by cytidine, as in

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mycoplasma tRNA^{Gly}). The tRNA$_1^{Gly}$-ASL was obtained by site-directed mutagenesis of the cloned tRNA$_1^{Gly}$(CCC) gene, essentially as described (14). The mutagenized tRNA$_1^{Gly}$-ASL gene was cloned under the *tac* promoter in expression vector pKK 223-3 (14).

The tRNA$_1^{Gly}$-AS and tRNA$_1^{Gly}$-C32 genes were constructed from the synthetic oligonucleotides, essentially as described by Sampson and Uhlenbeck (16). The DNA fragment, corresponding to the tRNA$_1^{Gly}$-C32 gene, was cloned into pTZ19R vector under the T7 promoter, and the DNA fragment, corresponding to the tRNA$_1^{Gly}$-AS gene with its own T7 promoter, was cloned into transcription vector pET-3. The recombinant vectors were further used for transformation of *E. coli* BL21(DE3)pLysS cells carrying the inducible gene for T7 RNA polymerase. Sequences of the above tRNA^{Gly} mutants are shown in Fig. 1.

Expression of tRNA$_1^{Gly}$ Mutants. Expression of tRNA$_1^{Gly}$(UCC) and tRNA$_1^{Gly}$-ASL was done as described (14). Expression of tRNA$_1^{Gly}$-C32 and tRNA$_1^{Gly}$-AS was as in ref. 17. The tRNAs were purified on benzoylated DEAE-cellulose, as described (14). When the pattern of isoaccepting tRNA^{Gly}s in cells transformed with recombinant vector was compared with that of cells with nonrecombinant vector, the mutated tRNA$_1^{Gly}$s were ≈20–50 times overexpressed compared with the host-cell tRNA$_1^{Gly}$.

Cloning and Expression of Mycoplasma tRNA^{Gly} Gene in *E. coli*. These techniques were done as described (18, 19).

Identification of Wobble Nucleotide. The purified tRNA$_1^{Gly}$ mutants and the tRNA^{Gly} from *M. mycoides* were sequenced by the Stanley and Vassilenko method (20) with some modifications (21). Results of the sequence analysis indicated that the wobble nucleotide of tRNA$_1^{Gly}$(UCC) and tRNA$_1^{Gly}$-C32 showed the mobility expected for unmodified uridine (Fig. 2), whereas tRNA$_1^{Gly}$-ASL and mycoplasma tRNA^{Gly} expressed in *E. coli* contained an unknown modification of uridine in this position (data not shown).

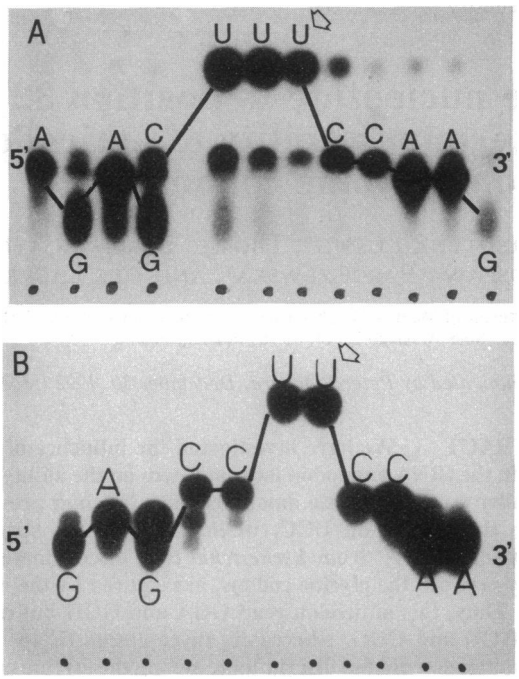

FIG. 2. Sequence analysis of tRNA^{Gly} mutants. The tRNA^{Gly} mutants were purified by two-dimensional gel electrophoresis, and sequences were determined by using a postlabeling technique (20, 21). The nucleotides were separated on a TLC plate with HCl/isopropanol/H_2O, 15:70:15 (wt/vol) as solvent. Wobble nucleotides are indicated by arrows. (*A*) tRNA$_1^{Gly}$(UCC). (*B*) tRNA$_1^{Gly}$-C32.

RESULTS

Codon-Reading Properties of tRNA$_1^{Gly}$ Mutants. For these experiments we used a protein-synthesizing system that strictly depended on added glycyl-tRNA (13). Four modified MS2 RNAs were used to program the *in vitro* system, one in which GGU was the only glycine codon in the coat-protein gene, another that had only GGC, etc. (14). The system produced a coat-protein fragment that could be used to monitor reading of the appropriate glycine codon. Conditions

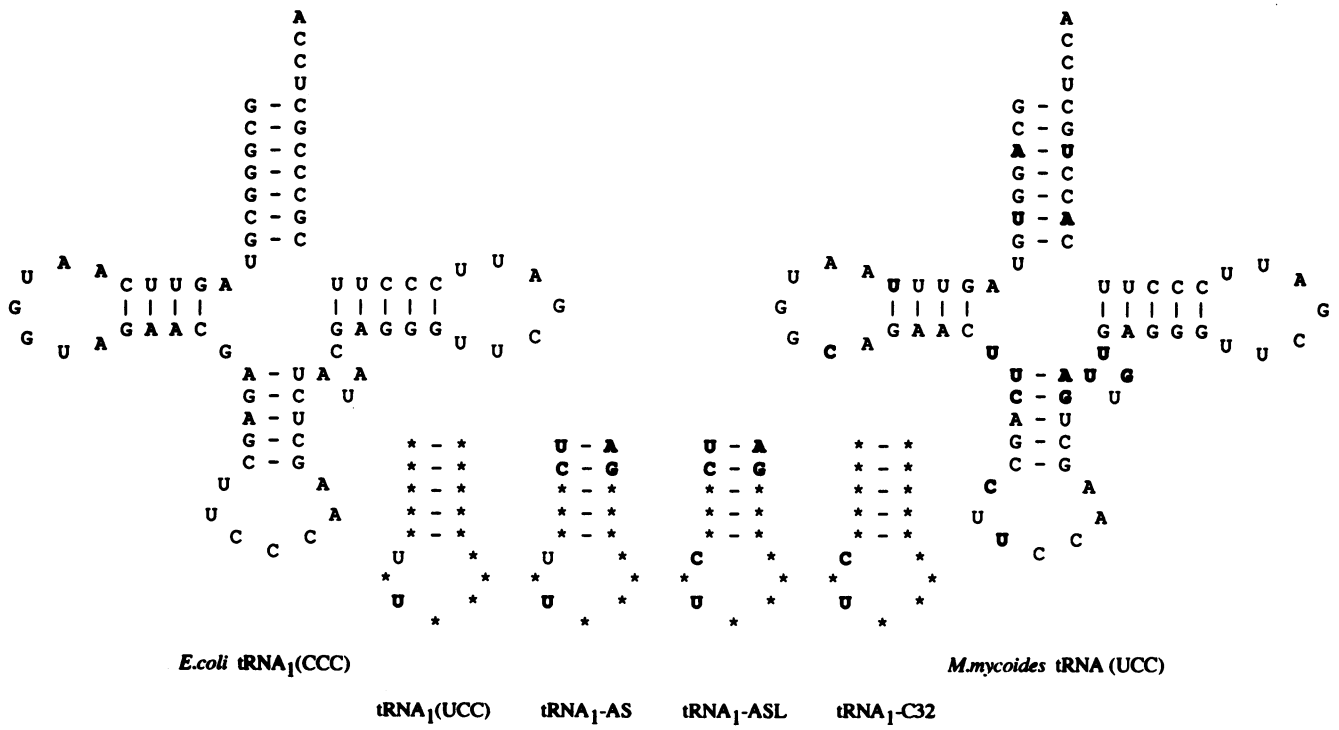

FIG. 1. Sequences of mutated tRNAs derived from tRNA$_1^{Gly}$(CCC) gene. Differences in mutants and in mycoplasma tRNA^{Gly} relative to tRNA$_1^{Gly}$ are indicated by boldface letters. Asterisks denote nucleotides identical with nucleotides in tRNA$_1^{Gly}$. Modifications are not shown.

for *in vitro* protein synthesis and purification of the coat-protein fragment were as described (14, 19).

To evaluate the relative reading efficiencies of different isoacceptor $tRNA^{Gly}$s, experiments were done in which two tRNAs competed with each other for the same codon. In a competing pair one tRNA was esterified with [^{3}H]glycine and the other was esterified with [^{14}C]glycine; the tRNAs were then incubated together in equimolar concentrations in the *in vitro* protein-synthesizing system. The relative efficiency of the competing tRNAs in reading a certain codon could be calculated by comparing the $^3H/^{14}C$ ratio in the coat-protein fragment, synthesized in response to the modified MS2 RNA containing the appropriate glycine codon, with the isotopic ratio of the glycyl-tRNAs incubated.

As a reference in our competition experiments we used the readily available mycoplasma $tRNA^{Gly}$ expressed in *E. coli* [$tRNA^{Gly}$ M(E)], which contains an unknown modification of uridine in the wobble position, rather than $tRNA^{Gly}$ from mycoplasma ($tRNA^{Gly}$ M) (19).

Previous sequence analysis of the construct $tRNA_1^{Gly}$(UCC) by using the Gupta and Randerath method (22) indicated that the wobble nucleotide was an unsubstituted uridine, consistent with the results of HPLC analysis that detected only the modified uridine phosphates present also in wild-type $tRNA_1^{Gly}$ (14). In Fig. 2*A* a faint additional spot moving as cytidine is seen corresponding to position 34, and similar additional spots are seen for positions 32 and 33 also. This finding is not constant and might represent an artifact, as it is unlikely that uridine in positions 32 and 33 should be modified. However, we cannot exclude the possibility that a small minority of the $tRNA_1^{Gly}$(UCC) molecules might contain an unknown modification of uridine in position 34. Nevertheless, the vast majority have unsubstituted uridine in this position, and the sequence results are consistent with our previous conclusion that anticodon UCC, when in the structural context of the $tRNA_1^{Gly}$ molecule, behaved as predicted by the wobble rules. On the other hand, in the structural context of mycoplasma $tRNA^{Gly}$ the anticodon read without discrimination among nucleotides in the third-codon position, in violation of wobble restrictions (14).

We next addressed the problem of how the structural context provided by the tRNA molecule for the anticodon could influence anticodon ability to discriminate among nucleotides in the third position of glycine codons. Specifically, we asked what influence the anticodon loop and stem might have in this respect. We, therefore, tested constructions $tRNA_1^{Gly}$-ASL, $tRNA_1^{Gly}$-AS, and $tRNA_1^{Gly}$-C32 (Fig. 1) for their ability to read glycine codons in our *in vitro* protein-synthesizing system. The results are summarized in Table 1 and Fig. 3.

In a series of experiments $tRNA_1^{Gly}$-ASL competed with $tRNA_1^{Gly}$(UCC) and the mycoplasma $tRNA^{Gly}$(UCC). The results showed that $tRNA_1^{Gly}$-ASL read GGU and GGC much better than $tRNA_1^{Gly}$(UCC) and almost as efficiently as mycoplasma $tRNA^{Gly}$(UCC) expressed in *E. coli*. In terms of their ability to read the codon GGA, where they comply with the wobble rules, these tRNAs did not differ significantly. These results lead to the conclusion that the structure of the

Table 1. Efficiency of $tRNA^{Gly}$s in reading glycine codons

	Relative reading efficiency of codon*							
	GGU		GGC		GGA		GGG	
Competing $tRNA^{Gly}$s	Exp.	Mean	Exp.	Mean	Exp.	Mean	Exp.	Mean
$tRNA_1^{Gly}$(UCC)/$tRNA^{Gly}$ M(E)	0.026		0.025		0.33		0.080	
	0.014		0.095		0.20		0.043	
	0.014	0.044	0.096	0.057	0.18	0.30	0.038	0.076
	0.060		0.065		0.27		0.047	
	0.094		0.038		0.42		0.15	
	0.058		0.025		0.40		0.099	
$tRNA^{Gly}$M/$tRNA^{Gly}$ M(E)	1.3		1.4		0.42		0.46	
	2.2		2.0		0.71		0.70	
	0.82	1.6	0.84	1.6	0.30	0.55	0.27	0.56
	2.2		2.0		0.77		0.81	
$tRNA_1^{Gly}$(UCC)/$tRNA_1^{Gly}$-ASL	0.084		0.056		0.46		0.11	
	0.056	0.055	0.046	0.040	0.35	0.36		0.073
	0.024		0.019		0.26		0.035	
$tRNA^{Gly}$M/$tRNA_1^{Gly}$-ASL	0.59		1.7		0.74			
	1.9	1.9		2.8	0.73	1.02	0.98	1.7
	3.1		3.8		1.6		2.5	
$tRNA_1^{Gly}$(UCC)/$tRNA_1^{Gly}$-AS	0.96		0.81		1.23		0.98	
	1.08	1.02	0.82	0.82	1.5	1.4	1.05	1.02
$tRNA_1^{Gly}$-AS/$tRNA^{Gly}$ M(E)	0.09		0.067		0.68		0.14	
	0.12	0.11	0.089	0.078	0.49	0.59	0.14	0.14
$tRNA_1^{Gly}$(UCC)/$tRNA_1^{Gly}$-C32	0.066		0.038		0.56		0.20	
	0.067	0.069	0.046	0.038	0.46	0.49	0.17	0.17
	0.073		0.030		0.46		0.14	
$tRNA_1^{Gly}$-C32/$tRNA^{Gly}$ M(E)	0.63		0.71		0.86		0.53	
	0.53	0.62	0.55	0.65	0.80	0.85	0.48	0.58
	0.69		0.69		0.89		0.72	
$tRNA_1^{Gly}$-C32/$tRNA_1^{Gly}$-ASL	0.86		0.98		0.70		0.48	
	0.81	0.84	0.96	0.95	0.65	0.64	0.44	0.42
	0.86		0.91		0.56		0.33	

*To determine relative reading efficiency, the two tRNAs esterified with [^{3}H]glycine and [^{14}C]glycine, respectively, were incubated together in the *in vitro* protein-synthesizing system programmed with the modified MS2 RNA messengers representing the different glycine codons. By comparing the $^3H/^{14}C$ ratio in the purified coat-protein fragment synthesized in response to the modified MS2 RNA containing the appropriate glycine codon with the isotopic ratio of the glycyl-tRNAs incubated, the relative efficiency of competing tRNAs in reading a certain codon could be calculated.

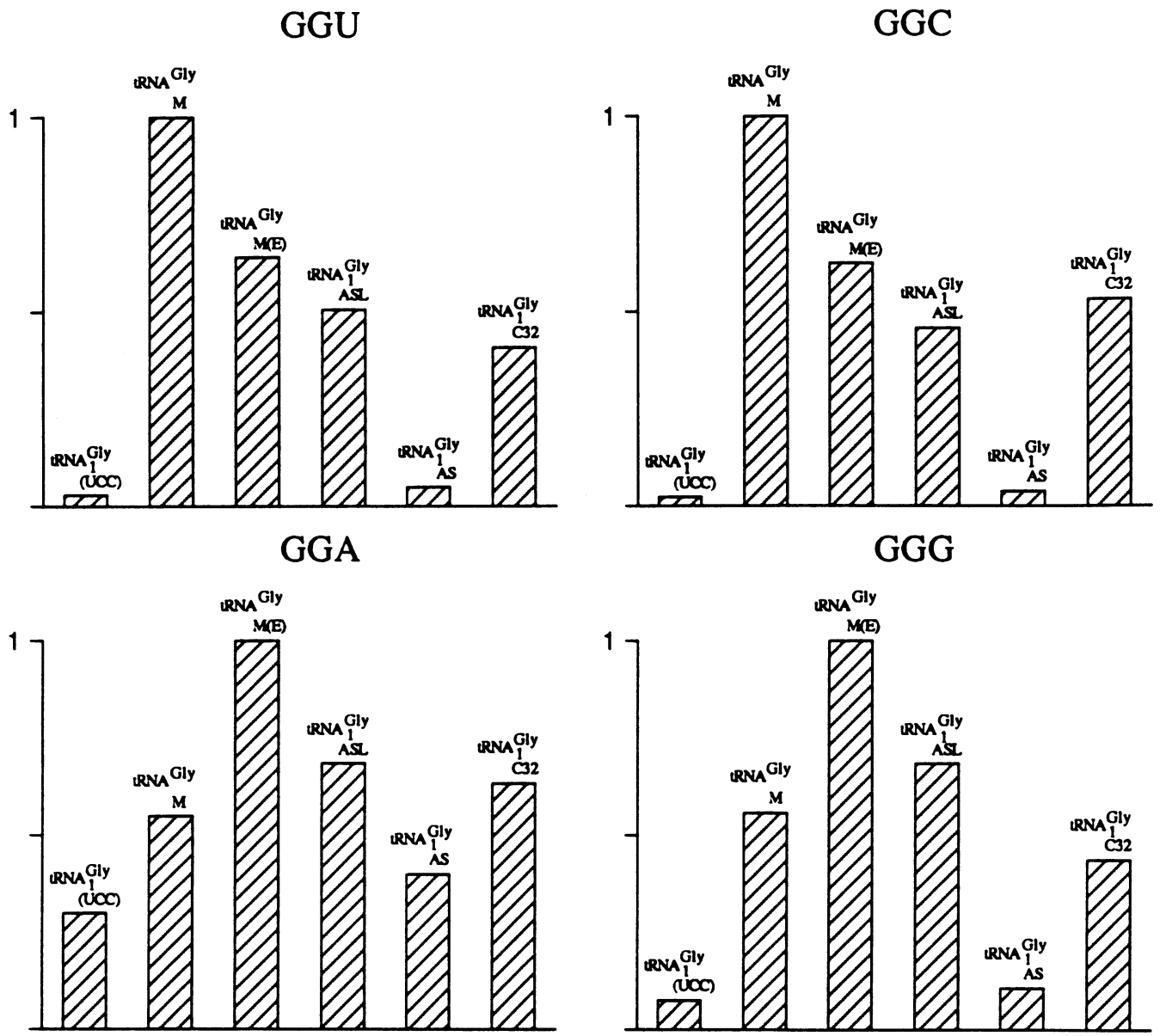

FIG. 3. Histograms showing relative reading efficiencies of different $tRNA^{Gly}$s. Reading efficiencies are given as mean values, with the highest efficiency used as an arbitrary unit. For further details see the footnote for Table 1 and *Materials and Methods*.

anticodon loop and stem is of decisive importance for the discriminating ability of anticodon UCC.

$tRNA_1^{Gly}$-ASL differs from $tRNA_1^{Gly}$(UCC) in positions 27,28 and 42,43 of the anticodon stem and in position 32 of the anticodon loop (Fig. 1). We next asked which of these nucleotides were important for the ability of the anticodon to discriminate. The competition experiments between $tRNA_1^{Gly}$-AS and other $tRNA^{Gly}$s show that this tRNA mutant discriminated among the nucleotides in the third position of glycine codons, as required by the wobble rules. Thus, changing the anticodon stem did not change the capacity of the tRNAs for discriminating reading.

The results of experiments where construct $tRNA_1^{Gly}$-C32 competed against mycoplasma $tRNA^{Gly}$, $tRNA_1^{Gly}$-ASL, and $tRNA_1^{Gly}$(UCC) showed that it was almost as efficient as mycoplasma $tRNA^{Gly}$ and $tRNA_1^{Gly}$-ASL in reading GGU and GGC. One must, therefore, conclude that it is the nature of nt 32 of a tRNA structure otherwise identical with $tRNA_1^{Gly}$(UCC) that determines whether or not the anticodon will discriminate among the glycine codons. With uridine in position 32 the tRNA discriminates perfectly well, but when the uridine is replaced by a cytidine, the tRNA loses its discriminatory power.

In reading GGA, all glycine $tRNA^{Gly}$s were about equally effective, whereas in terms of their ability to wobble and read GGG the mutants $tRNA_1^{Gly}$-ASL and $tRNA_1^{Gly}$-C32 were better than the very restricted wobbler $tRNA_1^{Gly}$(UCC). We, therefore, conclude that the structure of the anticodon loop and stem is important for the ability of anticodon UCC to wobble efficiently.

DISCUSSION

In most cases, where there is unrefutable evidence *in vivo* of a single tRNA reading all four codons in a family box—i.e., reading without discrimination in the third-codon position—and the primary structure of the tRNA is known, the tRNA has an unsubstituted uridine in the wobble position (23). It has been suggested (2) that family codons ending in uridine or cytidine may be read by a two-out-of-three mechanism in the mitochondrion, in which case the ubiquitous uridine in the

wobble position would have been evolutionarily selected because it forms the least-objectionable mispairs (24).

In our investigation we have attempted to determine the influence of structures in the anticodon loop and stem on UCC ability to discriminate among glycine codons. We have used three tRNA constructs based on $tRNA_1^{Gly}$(UCC), which we have called $tRNA_1^{Gly}$-ASL, $tRNA_1^{Gly}$-AS, and $tRNA_1^{Gly}$-C32 (Fig. 1). These constructs were tested for their ability to read glycine codons in experiments where they competed against each other or against the mutant $tRNA_1^{Gly}$(UCC) or mycoplasma $tRNA^{Gly}$(UCC) in our *in vitro* protein-synthesizing system. Our results showed that the anticodon UCC, when present in construct $tRNA_1^{Gly}$-ASL, where the anticodon loop and stem are the same as in mycoplasma $tRNA^{Gly}$ and the rest of the molecule is derived from $tRNA_1^{Gly}$, had lost its ability to discriminate among glycine codons, as prescribed by the wobble rules. At the same time, $tRNA_1^{Gly}$-AS, in which the anticodon stem is identical with mycoplasma $tRNA^{Gly}$, discriminated as efficiently as $tRNA_1^{Gly}$(UCC). In construct $tRNA_1^{Gly}$-C32, where anticodon UCC is present in a structural context derived entirely from $tRNA_1^{Gly}$, except that the uridine in position 32 was replaced by a cytidine, the anticodon still could not discriminate among glycine codons—i.e., it behaved as in mycoplasma $tRNA^{Gly}$.

These results strongly suggest that the nature of nt 32 of the anticodon loop decisively influences the reading properties of anticodon UCC. When position 32 is occupied by a uridine, this nucleotide confers on the anticodon the ability to discriminate among glycine codons, whereas with a cytidine in the same position the ability to discriminate is lost. In this context it is interesting to note that all tRNAs in *M. capricolum* and *M. mycoides* that can read each of the four codons in a family codon box have a cytidine in position 32, the only exception being $tRNA^{Pro}$, which has a uridine in this position (7–10).

By what mechanism could cytidine, when present at position 32 instead of uridine, deprive anticodon UCC of its ability to discriminate among glycine codons? One obvious hypothesis would be that a cytidine in this position affects the tRNA conformation and, in particular, the anticodon loop, so as to alter reading properties of the anticodon. For instance, it has been suggested that U32 might pair with A38 and that $tRNA_1^{Gly}$-C32 could be deficient in such a pairing.

An alternative hypothesis is that the ability to discriminate among third-position nucleotides of the glycine codons is mainly a question of kinetics and involves the contact time of tRNA on the ribosome. A cytidine, instead of a uridine, in position 32 might allow more stable interaction with, for instance, the 16S RNA, thus increasing contact time.

That contact time determines discrimination would in itself argue that indiscriminate reading of the glycine codons GGU and GGC by anticodon UCC cannot involve good base pairing between uridine in the anticodon wobble position and uridine or cytidine in the third-codon position. Were these mispairs stable, there would be no necessity for a prolonged contact time. In other words, such reading would have to be the two-out-of-three type (11).

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