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The discriminator base influences tRNA structure at the end of the acceptor stem and possibly its interaction with proteins

(aminoacyl-tRNA synthetase/Met-tRNA transformylase/peptidyl-tRNA hydrolase/elongation factor/RNA base-pair stability)

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ABSTRACT **For many tRNAs, the discriminator base preceding the CCA sequence at the 3′ end is important for aminoacylation. We show that the discriminator base influences the stability of the 1·72 base pair onto which it is stacked. Mutations of the discriminator base from adenosine to cytidine or uridine make the cytidine residue in the C1·G72 base pair of mutant *Escherichia coli* initiator tRNAs more reactive toward sodium bisulfite, the single-strand-specific reagent. The activity of the enzyme Met-tRNA transformylase toward these and other mutant initiator tRNAs is also consistent with destabilization of the 1·72 base pair *in vitro* and *in vivo*. By influencing the strength of the 1·72 base pair, the discriminator base could affect the energetic cost of opening the base pair and modulate the structure of the tRNA near the site of aminoacylation. For some aminoacyl-tRNA synthetases and other proteins that interact with tRNA, these factors could be important for specific recognition and/or formation of the transition state during catalysis.**

Nucleotide 73 preceding the CCA sequence common to all tRNAs is often called the discriminator base. This term was coined by Crothers *et al.* (1) who noted a correlation between the nature of this base in tRNA and the chemical nature of the amino acid specificity of the tRNA. A possible explanation proposed was that this nucleotide served as a discriminator site for aminoacyl-tRNA synthetases to subdivide tRNAs into groups for recognition purposes.

The importance of the discriminator base in aminoacyl-tRNA synthetase recognition of tRNAs is seen in the crystal structure of two aminoacyl-tRNA synthetase–tRNA complexes. Interestingly, the role of the same discriminator base, G73, in the two cases is different. In the *Escherichia coli* Gln-tRNA synthetase–$tRNA^{Gln}$ complex, G73 plays a structural role by stabilizing a form of tRNA structure needed for the CCA end of the $tRNA^{Gln}$ to fit into the catalytic pocket of Gln-tRNA synthetase (2). In the yeast Asp-tRNA synthetase–$tRNA^{Asp}$ interaction, the enzyme contacts the discriminator base directly (3). Further indication of a general role for the discriminator base in aminoacylation of most tRNAs comes from the finding that mutations in the discriminator base often affect aminoacylation kinetics (4–17) and sometimes aminoacylation specificity (18–20). In most cases, however, the overall effect of the discriminator base mutation depends on the nature of the mutation. For example, in *E. coli* initiator methionine tRNA, mutation of A73 to U73 has essentially no effect on aminoacylation kinetics whereas mutation to C73 or G73 has a significant effect (16). These results suggest that in many cases the role of the discriminator base may be structural and, therefore, quite subtle.

In our studies on formylation of mutant *E. coli* initiator tRNAs by Met-tRNA transformylase (21), we showed that one of the crucial requirements was a mismatch or a weak base pair between nt 1 and 72 at the end of the acceptor stem (21). tRNAs carrying the wild-type CXA mismatch or virtually any other mismatch (16, 21, 22) are good substrates for the formylating enzyme whereas tRNAs carrying strong base pairs such as C1·G72 or G1·C72 are extremely poor substrates. These results suggest that nt 1 and 72 must be unpaired for formylation to occur. Interestingly, the severe effect on formylation of having a C1·G72 base pair could be compensated for by a change of the neighboring discriminator base A73 to either cytidine or uridine but not guanosine (16). A likely explanation of this result is that the C1·G72 base pair, which is at the end of an RNA helix and may, therefore, have a tendency to "breathe," is normally stabilized by stacking of the neighboring base A73 on the 3′ side of the C1·G72 base pair. Change of A73 to a pyrimidine base, such as cytidine or uridine, could destabilize the C1·G72 base pair due to loss of this stacking interaction, as seen in studies with model oligonucleotides (23, 24). In other words, the nature of the discriminator base influences the stability of the terminal base pair in the acceptor stem of tRNAs. In this paper, we provide chemical and additional enzymatic evidence to support this general conclusion.

MATERIALS AND METHODS

tRNA Mutants. The mutants, obtained by oligonucleotide-directed site-specific mutagenesis, are named according to the base changes in the tRNA (16).

The mutant tRNA genes were cloned into PTZ19R (a pUC derivative) and used to transform *E. coli* TG1 (a K-12 derivative), *E. coli* AA7852 (pth^{ts}) carrying a temperature-sensitive mutation in peptidyl-tRNA hydrolase (25), or *E. coli* B105 (16).

Isolation and Purification of Mutant tRNAs. These were as described (26). The mutants analyzed in Tables 1 and 2 were expressed in *E. coli* B 105, which lacks the $tRNA_2^{fMet}$ species (27).

Assays for Formylation *in Vitro*. Assays for formylation and measurement of kinetic parameters in the formylation reaction were as described (21).

Detection of Mutant tRNAs by RNA Blot Hybridization. Mutant tRNAs isolated by phenol extraction under acidic conditions to preserve the ester linkage between the amino acid and the tRNA were subjected to polyacrylamide gel electrophoresis at pH 5.0 and 4°C (28). The various forms of the mutant tRNA (tRNA, aminoacyl-tRNA, or formylaminoacyl-tRNA) were detected by Northern blot hybridization using deoxyribooligonucleotide probes labeled at the 5′ end with ^{32}P (16).

Reaction of 5′-^{32}P-Labeled tRNA with Sodium Bisulfite and Analysis of the Products. The reaction consists of two steps: (*i*) the addition of bisulfite across the 5,6 double bond of

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cytidine followed by deamination to form 5,6-dihydrouridine 6-sulfonate and (*ii*) the elimination of sulfonic acid from 5,6-dihydrouridine 6-sulfonate resulting in the overall conversion of cytidine to uridine (29, 30).

The tRNAs were labeled at the 5′ end with ^{32}P and purified by gel electrophoresis. The labeled tRNAs (≈15 μg) were incubated with 3 M $NaHSO_3$ and 10 mM $MgCl_2$ at pH 6.0 and at 23°C for the times indicated. The reaction mixtures (10 μl) were diluted to 100 μl and excess $NaHSO_3$ was removed by centrifuging the solution through a Sephadex G-50 spin column. tRNAs were recovered by precipitation with ethanol. The tRNAs were then incubated in 0.1 M Tris·HCl (pH 9.0) at 37°C for 8 h and digested to completion with RNase T2. The radioactive nucleoside diphosphates, [^{32}P]Cp and [^{32}P]Up, were separated by thin layer chromatography on plastic-backed cellulose plates using isobutyric acid/NH_4OH/H_2O, 66:1:33 (vol/vol), as the solvent. The plate was dried and autoradiographed, the radioactive spots corresponding to pCp and pUp were excised, and radioactivity was measured.

RESULTS

Effect of Discriminator Base Mutations on the Stability of an Adjacent C1·G72 Base Pair. We analyzed the effect of changes in the discriminator base on reactivity of the cytidine in the C1·G72 base pair of *E. coli* initiator mutants (Fig. 1) toward sodium bisulfite, the single-strand-specific reagent (29). This reagent reacts with cytidines that are unpaired and unstacked (30). The cytidine in the wild-type tRNA, which is part of a C1×A72 mismatch, is quite reactive. When the cytidine is part of a C1·G72 base pair as in the G72 mutant, it is much less reactive. Introduction of further mutations in the neighboring discriminator base shows that mutations of A73 to C73 or U73 result in increased reactivity of C1 toward sodium bisulfite. Mutation to G73 does not have such an effect.

Effect of Discriminator Base Mutations on the Stability of an Adjacent G1·C72 Base Pair. The above results suggest that the nature of the discriminator base can affect the stability of the C1·G72 base pair in tRNAs. Most tRNAs, however, have a G1·C72 base pair (31). Therefore, we examined whether the stability of a G1·C72 base pair in tRNAs is also influenced by the nature of the discriminator base. Based on studies with some model oligonucleotides, a G·C base pair at the end of an RNA helix is thought to be more stable than a C·G base pair (23).

Two mutants of *E. coli* initiator tRNA were studied: one with a G1·C72 base pair (16) and the other with an additional mutation of A73 → U73. Since tRNAs with weak or disrupted base pairs between nt 1 and 72 are better substrates for formylation, we used formylation of the mutant tRNAs *in vitro* and *in vivo* (28) as an indicator of the relative stability of the 1·72 base pair. As shown before (16, 22), the G1·C72 mutant with a strong 1·72 base pair is an extremely poor substrate for Met-tRNA transformylase (Fig. 2 and Table 1). This effect is almost exclusively due to an effect on V_{max}, indicating that the step affected is not binding but catalysis. Introduction of an additional U73 mutation, however, converts it into a better substrate.

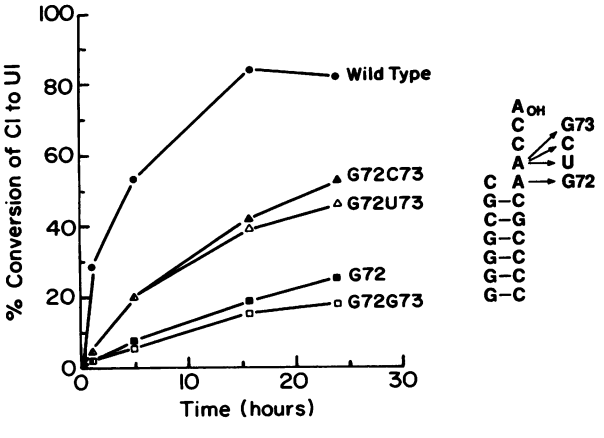

FIG. 1. Analysis of sodium bisulfite-mediated deamination of the 5′-terminal cytidine to uridine in wild-type and mutant initiator tRNAs.

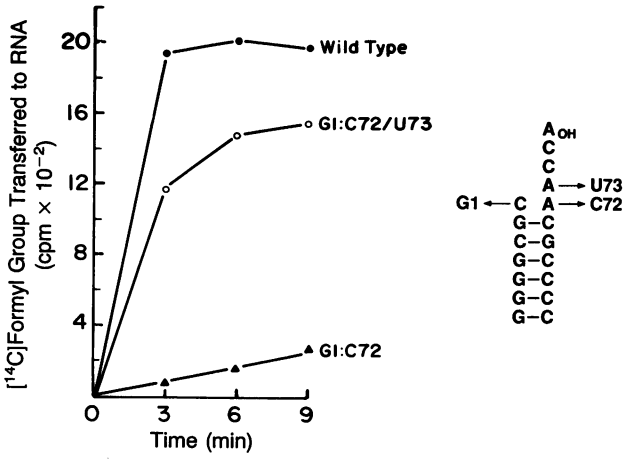

FIG. 2. Time course of incorporation of the [^{14}C]formyl group from N^{10}-[^{14}C]formyl-tetrahydrofolate into wild-type and mutant *E. coli* tRNAs. Assays for formylation were as described (21).

The results of *in vitro* analyses are also confirmed *in vivo* (Fig. 3). In contrast to the G1·C72 mutant, which is aminoacylated but not formylated, the G1·C72/U73 mutant is partially formylated (compare lanes 2 and 4). The presence of significant amounts of aminoacyl-tRNA for the G1·C72/U73 mutant agrees with the results of *in vitro* studies showing that while the U73 mutation can compensate for the severe negative effect on formylation of a G1·C72 base pair, it can do so only partly (Table 1).

Relative Stabilities of C1·G72 and G1·C72 Base Pairs in tRNA. It is interesting to compare the effect of U73 mutation on tRNA with a C1·G72 base pair to that with a G1·C72 base pair. The C1·G72/U73 mutant tRNA is a better substrate for Met-tRNA transformylase than the G1·C72/U73 mutant. The V_{max}/K_m in formylation for the C1·G72/U73 mutant is only 3.7-fold lower (16) than for the wild-type tRNA whereas that for the G1·C72/U73 mutant is 60-fold lower (Table 1). *In vivo*, the C1·G72/U73 mutant tRNA is essentially completely formylated (16), whereas the G1·C72/U73 is only partly formylated (Fig. 3, lane 2). These results suggest that C1·G72 base pair at the end of an RNA helix is more easily disrupted by Met-tRNA transformylase than a G1·C72 base pair and support the conclusions, based on thermodynamic measurements, that a G·C base pair at the end of an RNA helix contributes more to helix stability than a C·G base pair (23, 24).

Table 1. Kinetic parameters in formylation of mutant tRNAs carrying changes at positions 1, 72, and 73

tRNA	Relative V	K_m^{app}, μM	Relative V_{max}/K_m^{app}
$tRNA_2^{fMet}$	370	7.2	1
G1·C72	0.64	12.8	1035
G1·C72/U73	11.34	12.6	60

Relative V_{max}/K_m^{app} is the ratio of V_{max}/K_m^{app} of $tRNA_2^{fMet}$ to V_{max}/K_m^{app} of each mutant tRNA.

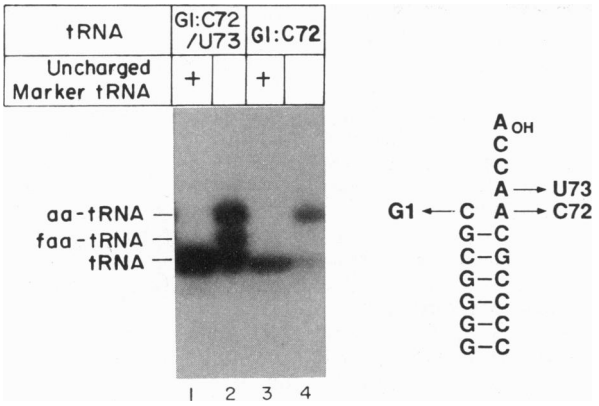

FIG. 3. Northern blot analysis of total tRNA isolated under acidic conditions from *E. coli* TG-1 transformants carrying the G1·C72 and G1·C72/U73 mutant initiator tRNA genes (28). The blot was probed with a ^{32}P-labeled oligonucleotide complementary to *E. coli* $tRNA_2^{fMet}$. Locations of uncharged tRNA, fMet-tRNA, and Met-tRNA are indicated. aa, Met.

An enzyme whose activity on a tRNA substrate depends upon the presence of a base pair between nt 1 and 72 is peptidyl-tRNA hydrolase (16, 32, 33). Its activity on the C1·G72/U73 and the G1·C72/U73 mutant tRNAs provides further indication that the C1·G72 base pair is more easily disrupted than a G1·C72 base pair. For example, in contrast to the C1·G72/U73 tRNA, essentially all of which is formylated (16), a substantial fraction of the G1·C72/U73 mutant tRNA is present as uncharged tRNA (Fig. 3, lane 2). Both mutants are equally good substrates for Met-tRNA synthetase (V_{max}/K_m^{app} down only by a factor of 2–3 compared to wild-type tRNA). Therefore, the most likely reason for accumulation of uncharged tRNA for the G1·C72/U73 mutant *in vivo* is that, as for the U1 mutant (previously called T1) (16), the fMet-tRNA corresponding to the G1·C72/U73 mutant is a substrate for peptidyl-tRNA hydrolase, which hydrolyzes it to fMet and tRNA. This is supported by the finding that, in strains carrying a temperature-sensitive mutation in peptidyl-tRNA hydrolase (25), there is virtually no accumulation of uncharged G1·C72/U73 tRNA even at 30°C, the permissive temperature (compare Fig. 4, lane 2, with Fig. 3, lane 2). Thus, *in vivo*, most of the C1·G72/U73 mutant tRNA exists in a form in which the C1·G72 base pair is broken, whereas a good fraction of the G1·C72/U73 mutant

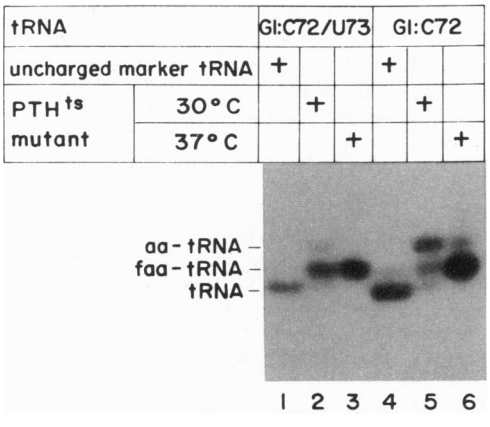

FIG. 4. Northern blot analysis of total tRNA isolated under acidic conditions from *E. coli* AA7852 (pth^{ts}, ref. 25) transformants, grown at 30°C and 37°C, carrying the G1·C72 and G1·C72/U73 mutant initiator tRNA genes. The blot was probed with the same oligonucleotide indicated in Fig. 3. Locations of uncharged tRNA, fMet-tRNA, and Met-tRNA are indicated. aa, Met.

Table 2. Effect of changes in the discriminator base on formylation of mutant tRNAs

tRNA	Relative V_{max}/K_m^{app}
$tRNA_2^{fMet}$	1.0
U73	2.1
C73	1.1
G73	8.1

exists in a form in which G1 and C72 are base-paired. The accumulation of the G1·C72 mutant as fMet-tRNA at 37°C (Fig. 4, lane 6) is due to the fact that at this temperature, protein synthesis essentially comes to a halt (25) and the end product, fMet-tRNA, accumulates although the rate of formylation of this mutant tRNA is extremely low.

Effect of Discriminator-Base Mutations on Recognition of *E. coli* Initiator tRNA by Met-tRNA Synthetase and Met-tRNA Transformylase. Mutations of A73 to G73 or C73 have a detrimental effect on aminoacylation whereas mutation to U73 has no effect (16). Since A73 and U73 have no functional groups in common, the effect of A73 to G73 or C73 mutations is most likely due to unfavorable interactions and/or alteration of tRNA structure at the end of the acceptor stem rather than loss of a contact site for Met-tRNA synthetase.

Studies on formylation of the same mutant tRNAs by Met-tRNA transformylase show that while the G73 mutation lowers the V_{max}/K_m parameter by a factor of ≈8 (Table 2 and ref. 22), mutations to C73 and U73 have little effect. Therefore, for Met-tRNA transformylase also, it is unlikely that the discriminator base is a site of direct contact. The detrimental effect, specifically, of G73 could be due to an alteration in local structure of the tRNA. Since nt 1 and 72 in the *E. coli* initiator tRNA are unpaired (34), the G73 mutant could adopt a "hairpinned" structure on its own, similar to that of $tRNA^{Gln}$ in the *E. coli* Gln-tRNA synthetase–$tRNA^{Gln}$ complex (2). The G73 mutation may thus exemplify a mutation in the discriminator base that alters local structure of the tRNA and, consequently, affects its interaction with an enzyme, Met-tRNA transformylase.

DISCUSSION

The conclusion that the nature of the discriminator base influences the stability of the terminal 1·72 base pair in the acceptor stem does not mean that this base pair is actually melted in the tRNA. Rather, the presence of a pyrimidine instead of a purine in the discriminator position reduces the strength of the 1·72 base pair (23, 24), thereby lowering the energetic cost of opening the base pair (35). Such a "flexibility" in the structure of the free tRNA could allow a protein to use part of the binding energy to open the 1·72 base pair for specific recognition and/or formation of the transition state during catalysis. Strong support for this possibility comes from the finding that an A73 → U73 mutant of *E. coli* tyrosine suppressor tRNA, which has a G1·C72 base pair, is now partly aminoacylated with glutamine in *E. coli* (20). Since *E. coli* Gln-tRNA synthetase disrupts the 1·72 base pair for aminoacylation (2), mutation of A73 to U73 must facilitate disruption of the G1·C72 base pair.

The energetic cost of opening the 1·72 base pair will be a function of the nature of the 1·72 base pair, the neighboring 2·71 base pair (24), and the discriminator base. Comparison of results obtained with the C1·G72/U73 and G1·C72/U73 mutants of *E. coli* initiator tRNA (cf. Fig. 3 with figure 5 of ref. 16 and Table 1 with table 4 of ref. 16) supports previous conclusions based on model oligonucleotides that a G·C base pair at the end of an RNA helix is more stable than a C·G base pair (23). While most tRNAs (36 of 46) in *E. coli* have a G1·C72 base pair, proline tRNAs have a C1·G72 base pair and

asparagine, glutamine, isoleucine, and tryptophan tRNAs have "weaker" base pairs such as U1·A72 or A1·U72 (36). Also, among tRNAs that have G·C base pairs at the end of the acceptor stem, histidine, glycine, and cysteine tRNAs have a "destabilizing" pyrimidine base on the 3′ side of the G·C base pair. Thus, there is a good potential for some aminoacyl-tRNA synthetases to discriminate among tRNAs based on the propensity of the 1·72 base pair to be disrupted. This propensity does not mean, however, that the 1·72 base pair will always be disrupted by a protein. Although *E. coli* $tRNA^{Gln}$ and yeast $tRNA^{Asp}$ both have the same U1·A72 base pair and G73 in the discriminator position, in the *E. coli* Gln-tRNA synthetase–$tRNA^{Gln}$ complex, the base pair is disrupted, whereas, in the yeast Asp-tRNA synthetase–$tRNA^{Asp}$ complex, it is not disrupted and the enzyme makes contact with this base pair (2, 3). What happens to the 1·72 base pair, therefore, depends on the tRNA, the protein, and the way in which the protein interacts with the tRNA (37).

The influence of the discriminator base on tRNA structure could have important implications in interpretation of results of discriminator base mutations on aminoacyl-tRNA synthetase recognition of tRNAs. Mutations in the discriminator base usually affect aminoacylation kinetics. For *E. coli* Ala-tRNA synthetase, mutations in the discriminator base have been shown to specifically affect the step involving transfer of the amino acid from a preformed Ala–AMP–enzyme complex to tRNA (13). The effect of discriminator base mutations on aminoacylation is often assumed to be due to loss of a contact site for the enzyme. However, for some tRNAs, the effect could also be due to altered structure of the tRNA or stability of the 1·72 base pair near the site of aminoacylation.

Another implication of our results is that the seemingly distinct modes of interaction of tRNAs with aminoacyl-tRNA synthetases based on sequence comparisons of corresponding prokaryotic and eukaryotic tRNAs may not be correct. As mentioned above, the *E. coli* Gln-tRNA synthetase breaks the U1·A72 base pair during its interaction with $tRNA^{Gln}$. This base pair is common to all eubacterial glutamine tRNAs (31), implying a common mode of interaction of eubacterial Gln-tRNA synthetase with $tRNA^{Gln}$. Interestingly, yeast and all eukaryotic glutamine tRNAs have a G1·C72 base pair instead of a U1·A72 base pair, and yeast Gln-tRNA synthetase does not aminoacylate *E. coli* $tRNA^{Gln}$ and vice versa. This would imply, at first glance, that the mode of interaction of yeast Gln-tRNA synthetase with its cognate $tRNA^{Gln}$ is different from that of *E. coli* Gln-tRNA synthetase with its cognate tRNA. However, the discriminator base in all the eukaryotic $tRNA^{Gln}$ species is uridine, which would render the G1·C72 base pair less stable than if the discriminator base was adenosine. Therefore, it is possible that, as with the U73 mutant of *E. coli* tyrosine suppressor tRNA mutant discussed above (20), the G1·C72 base pair is disrupted also during the interaction of yeast Gln-tRNA synthetase with $tRNA^{Gln}$.

Finally, the effect of mutations on the efficiency of suppression of termination codons by a tRNA is often used as a measure of aminoacylation of tRNAs *in vivo*. Mutations in the discriminator base do affect levels of suppression implying a role of the discriminator base in aminoacylation. However, it is important to also recognize the possible effect of such mutations on the affinity of the mutant aminoacyl-tRNA toward elongation factor Tu (38), which varies with the presence or absence of a base pair at the end of the acceptor stem (39).

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