RNA (1996), 2:919–927. Cambridge University Press. Printed in the USA.

Selection of tRNA^{Asp} amber suppressor mutants having alanine, arginine, glutamine, and lysine identity

FRANCK MARTIN, JOSEPH REINBOLT, GUY DIRHEIMER, JEAN GANGLOFF, and GILBERT ERIANI

Unité Propre de Recherche 9002 du Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, 67084 Strasbourg, France

ABSTRACT

Elements that confer identity to a tRNA in the cellular environment, where all aminoacyl-tRNA synthetases are competing for substrates, may be delineated by in vivo experiments using suppressor tRNAs. Here we describe the selection of active *Escherichia coli* tRNA^{Asp} amber mutants and analyze their identity. Starting from a library containing randomly mutated tRNA$^{Asp}_{CUA}$ genes, we isolated four amber suppressors presenting either lysine, alanine, or glutamine activity. Two of them, presenting mainly alanine or lysine activity, were further submitted to a second round of mutagenesis selection in order to improve their efficiency of suppression. Eleven suppressors were isolated, each containing two or three mutations. Ten presented identities of the two parental mutants, whereas one had switched from lysine to arginine identity. Analysis of the different mutants revealed (or confirmed for some nucleotides) their role as positive and/or negative determinants in AlaRS, LysRS, and ArgRS recognition. More generally, it appears that tRNA^{Asp} presents identity characteristics closely related to those of tRNA^{Lys}, as well as a structural basis for acquiring alanine or arginine identity upon moderate mutational changes; these consist of addition or suppression of the corresponding positive or negative determinants, as well as tertiary interactions. Failure to isolate aspartic acid-inserting suppressors is probably due to elimination of the important G34 identity element and its replacement by an antideterminant when changing the anticodon of the tRNA^{Asp} to the CUA triplet.

Keywords: DHFR; genetic selection; identity; suppression; tRNA^{Asp}

INTRODUCTION

Transfer RNAs play an essential role in the accurate decoding and translation of genetic information into amino acid sequences. This is achieved both by precise base pairing between the mRNA codon and tRNA anticodon, and by correct charging of the tRNA by its cognate aminoacyl-tRNA synthetase (aaRS). The amino acid acceptor specificity or identity of each tRNA is determined by a characteristic set of nucleotides and structural features called identity elements (reviewed by Normanly & Abelson, 1989; Schulman, 1991; Giegé et al., 1993; McClain, 1993a, 1993b; Saks et al., 1994). The identity set of a tRNA is composed of positive elements (determinants) that allow recognition by one aaRS, and negative elements (antideterminants) that prevent recognition by all others. Although extensive regions of the tRNA interact with the enzyme, only a small number of nucleotides determine the tRNA identity. Most of these are located in the amino acid acceptor helix region and the anticodon loop; exceptions are tRNA^{Ser}, tRNA^{Leu}, and tRNA^{Ala}, which are not recognized by their anticodons. Position 73 at the acceptor end, which is referred to as the discriminator base, has been shown to contribute to the identity of virtually every tRNA species. This particular base is also known in some cases to promote nonproductive interactions with noncognate aaRS (Uemura et al., 1982) or to stabilize the transition state of the acylation reaction step (Labouze & Bédouelle, 1989; Shi & Schimmel, 1991); its important contribution to the acceptor arm structure (Lee et al., 1993; Limmer et al., 1993) and its close proximity to the active site account for the strong influence of this base on the k_{cat}.

Identity also depends on primary structural features, such as the extra-arm of tRNA^{Ser} (Asahara et al., 1994), the extra base pair −1·73 of tRNA^{His} (Himeno et al., 1989), or nucleotides (acting indirectly) that establish

Reprint requests to: Gilbert Eriani, Unité Propre de Recherche 9002 du Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, 67084 Strasbourg, France; e-mail: eriani@ibmc.u-strasbg.fr.

a structural scaffold allowing optimal presentation of other identity elements (Quigley & Rich, 1976). These tertiary intramolecular interactions are well documented in the case of PheRS, CysRS, and ProRS (Sampson et al., 1990; Hou et al., 1993; McClain, 1993c; Hou, 1994; McClain et al., 1994).

Several in vitro and in vivo methodological advances have been developed that allow experimental description of identity elements. In vitro assays analyze the effect of mutations on the aminoacylation of tRNA transcripts obtained by run off transcription of synthetic tRNA genes (Sampson & Uhlenbeck, 1988). These experiments, however, do not show the effects of aaRS competition; hence, the results reflect only the consequence of tRNA recognition by the cognate aaRS, not competition by the other aaRS. Moreover, tRNA transcripts have no posttranscriptional base modifications, whereas these were shown to enhance structural stability of the tRNAs or in some cases function as positive or negative identity elements: lysidine at position 34 in $tRNA^{Ile2}_{CAU}$, for example, is recognized by IleRS (Muramatsu et al., 1988). Likewise, the first nucleotide of the $tRNA^{Glu}$ anticodon mnm^5s^2U is a determinant for aminoacylation by GluRS (Sylvers et al., 1993). On the contrary, the $_{m1}G$ at position 37 of yeast $tRNA^{Asp}$ acts as an antideterminant against recognition by ArgRS (Pütz et al., 1994).

Complete identity sets may only be determined by in vivo approaches using, for instance, nonsense suppressor tRNAs derived from synthetic genes expressed in bacterial cells (Normanly et al., 1986). In this case, the accepting identity of the suppressor tRNA is established by determining which amino acid it incorporates at an amber codon positioned near the N terminus of the dihydrofolate reductase reporter protein (DHFR). This requires purification and sequencing of the reporter protein.

Amber suppressors have been constructed for most of the *Escherichia coli* tRNA isoacceptors. Among the 23 tRNAs, 11 retained their identity despite having a different amber anticodon. Six tRNA became lysine-inserting suppressors and five suppressors accepted glutamine (Kleina et al., 1990; Normanly et al., 1990). Replacement of anticodons of $tRNA^{Pro,Thr1,Glu,Asp,Asn}$ by an amber triplet renders them completely inactive. However, active suppressors could be obtained from these inactive tRNAs by mutational changes in their sequences (Kleina et al., 1990; Normanly et al., 1990). Among them, the amber suppressor $tRNA^{Asn}$ was isolated recently, using a genetic screen based on suppression of two reporter proteins (Martin et al., 1995).

The present paper reports the isolation of amber $tRNA^{Asp}$ suppressors by in vivo selection. Fifteen active amber $tRNA^{Asp}$ mutants were obtained by genetic screening, presenting four different amino acid acceptor identities and variable levels of suppression efficiencies. The suppression efficiency reflects the amount of charged tRNA and its ribosomal performance. Mutants analysis assessed (or confirmed for some nucleotides) their role as identity elements. More generally, this study uncovered identity potentialities present in $tRNA^{Asp}$ and emphasizes the importance of an appropriate tertiary structure for revealing them.

RESULTS AND DISCUSSION

Background

Amber $tRNA^{Pro,Thr1,Glu,Asp,Asp}$ require sequence modifications to become active suppressors in vivo. No active suppressors were available for $tRNA^{Asn}$ (Kleina et al., 1990; Normanly et al., 1990) until recently, when glutamine-accepting mutant $tRNA^{Asn}_{CUA}$ was isolated by genetic selection (Martin et al., 1995). Previous experiments with $tRNA^{Asp}_{CUA}$ showed that it may be charged in vivo by LysRS after being mutated at not less than five positions in the anticodon loop and arm (Normanly et al., 1990). In the present study, we screened extensively for further *E. coli* amber suppressor $tRNA^{Asp}$ variants by a genetic screen similar to the one used for selection of $tRNA^{Asn}$ suppressors (Martin et al., 1995). These suppressors are valuable tools for determination of the structural elements defining the $tRNA^{Asp}$ identity in vivo. Another interest of this study is that functionally compensating mutations for the inactive $tRNA^{Asp}_{CUA}$ may contribute to exploration of tRNA identity elements. Active $tRNA^{Asp}_{CUA}$ mutants were selected from a library of amber $tRNA^{Asp}$ mutants obtained by PCR amplification under mutagenic conditions (Leung et al., 1989). The indicator *E. coli* strain 121R, which contains two amber mutations, the *argE*, encoding *N*-acetylornithinase involved in arginine biosynthesis, and the fusion *lacI-Z*, encoding β-galactosidase, was used for the screen. For this strain, growth is restricted on minimal medium (M9 medium) in the absence of suppression of the *argE* gene. Similarly, the blue coloration of colonies (resulting from transformation of the chromogenic substrate X-gal) only appears when suppression of the *lacI-Z* amber mutation occurs. It was found that the *argE* phenotype is more sensitive than the *lacI-Z* phenotype. Thus, for low suppression levels only white colonies appear, whereas normal-sized blue colonies represent higher levels.

Four positions are critical for suppressor activity of $tRNA^{Asp}_{CUA}$

Two-thousand clones from a library of randomly mutated $tRNA^{Asp}_{CUA}$ transformed into *E. coli* were screened for growth on M9 X-gal medium. Small white colonies and blue colonies were obtained at 37 °C within 24–72 h. To examine whether the phenotypes were related to amber suppression by the selected tRNA, recombinant plasmid DNAs were extracted from the colonies and

used to retransform strain 121R grown on the same medium. Finally, the amber suppressor tRNA genes were sequenced. Sequencing revealed that all blue colonies bore an amber suppressor tRNA^{Asp} with a C38A mutation; white colonies contained an A3G or G10A mutation, or a deletion of C56 (ΔC56) (Table 1; Fig. 1). Suppression efficiencies were measured in the *lacI-Z$_{am}$* suppression context and were compared to the basal *lacI-Z$^+$* activity of strain UF261. The relative values obtained were about 0.09% for the C38A mutant, 0.06% for A3G and G10A mutations, and 0.03% for the ΔC56 variant. Identity of each suppressor tRNA has been determined by co-expressing its corresponding gene together with the *fol$_{am}$* gene and sequencing of the purified DHFR protein resulting from suppression. The most active suppressor, C38A, inserted lysine 83% of the time, but also glutamine 17% of the time. The A3G mutant was mainly an alanine-inserting suppressor in addition to 26% lysine, whereas G10A and ΔC56 variants inserted exclusively lysine and glutamine, respectively (Table 1; Fig. 1). These results may be interpreted in light of what we know about identity elements of the corresponding specific tRNAs.

It is worth noting that some determinants corresponding to the above mentioned identities already are present in tRNA$^{Asp}_{CUA}$ (Fig. 2); these include the alanine identity element G2·C71 (McClain et al., 1991; Francklyn et al., 1992), as well as U35, which is an important positive determinant common to the anticodons of tRNA^{Gln,Lys} for GlnRS and LysRS recognition. In addition, tRNA$^{Asp}_{CUA}$ shares a second identity element with tRNA^{Lys}, C34; this may be the reason why the lysine identity is predominant among all analyzed mutants. Despite this favorable context, tRNA$^{Asp}_{CUA}$ remains inactive, and additional mutations are required in order to be recognized efficiently by LysRS, AlaRS, or GlnRS. These mutations may create new determinants or eliminate antideterminants, but may also modify the structural scaffold of the tRNA in a way to bring potential identity elements in productively interacting positions with the corresponding aaRS.

Less surprising was the alanine acceptor capacity appearing with the A3G mutation, because this substitu-

TABLE 1. Identities and suppression efficiencies of in vivo-selected tRNA$^{Asp}_{CUA}$ mutants.[a]

tRNA mutation	Suppression efficiency (%)	Amino acid acceptance	Colonies' phenotype
Native	nm	nd	
A3G	0.07	Ala 74%, Lys 26%	Arg+
G10A	0.06	Lys	Arg+
C38A	0.1	Lys 83%, Gln 17%	Arg+ Lac+
ΔC56	0.03	Gln	Arg+

[a] Suppression efficiencies are expressed as percentages of β-galactosidase activity induced by a mutant tRNA$^{Asp}_{CUA}$ in strain 121R relative to the β-galactose activity of the standard strain UF261. Activities were measured in a luminometer using the LumiGAL kit (Clontech); accuracy of the measurements is within ±10%. Amino acid acceptance of the suppressors was established by sequencing the corresponding "suppressed" DHFR protein purified previously in one step by affinity chromatography. The colonies' phenotype refers to suppression of the *argE$_{am}$* and *lacI-Z$_{am}$* genes on M9 medium supplemented with IPTG and X-gal. Colonies [Arg$^+$ Lac$^-$] are white, colonies [Arg$^+$ Lac$^+$] are blue.

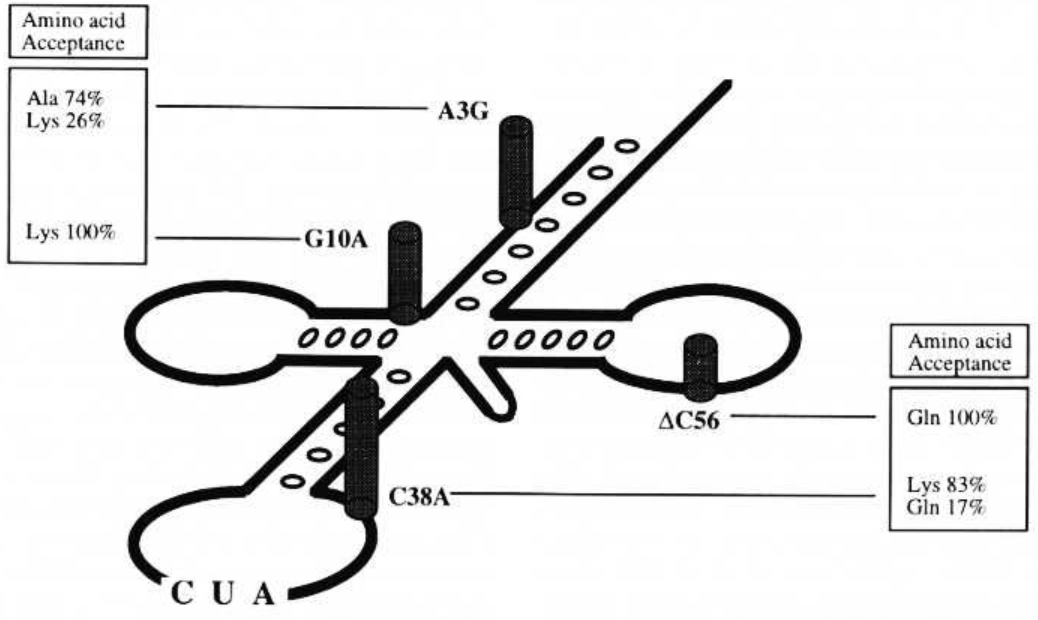

FIGURE 1. tRNA^{Asp} amber suppressors isolated by genetic screening from a library containing randomly mutated tRNA$^{Asp}_{CUA}$. Rates of amino acids incorporated in the DHFR protein by each suppressor are indicated in the front of the corresponding mutations. Heights of the cylinders reflect approximately the relative importance of the suppressors efficiencies.

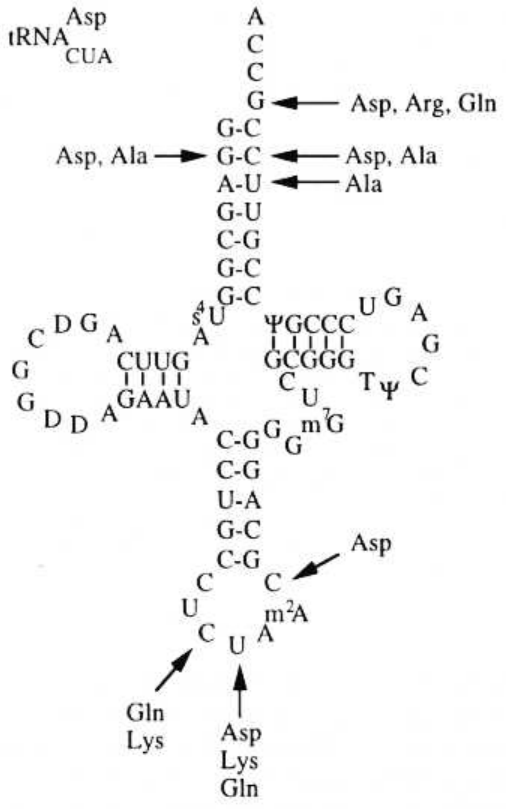

FIGURE 2. Cloverleaf representation of the $tRNA^{Asp}_{CUA}$ nucleotide sequence showing identity determinants present in the starting molecule before mutagenesis.

tion leads to the G3·U70 base pair, which is the major identity element of $tRNA^{Ala}$ (Hou &, Schimmel, 1988; McClain & Foss, 1988a). Introduction of this base pair into $tRNA^{Phe,Lys,Tyr}$ was already shown to confer alanine acceptance (Hou & Schimmel, 1988). It is noteworthy that neither the anticodon nor other parts of $tRNA^{Ala}$ are involved significantly in AlaRS recognition, with the exception of the discriminator base, the G2·C71 base pair, and the nucleotide G20, which, however, only play a minor role. This might suggest that the alanine identity can be acquired by a noncognate tRNA much more easily than any other identity, because it only requires a limited number of determinants.

More interesting was the partial lysine identity (26%) displayed by the A3G mutant. Despite containing the strong alanine determinant, it remained an efficient substrate for LysRS even when AlaRS was present. This lysine acceptance may not be ascribed to creation of new contacts at position 3·70 upon A3G mutation because LysRS was not shown to interact with these positions in $tRNA^{Lys}$ (McClain et al., 1990). More likely, creation of the G3·U70 wobble base pair might, as suggested, modify helicity of the acceptor arm (McClain et al., 1988) thereby bringing its 3′ end into a productive position with respect to the active site of LysRS. This structural modification could depend on the neighboring G4·U69 pair (Fig. 2).

Mutation G10A confers exclusively lysine identity to the amber suppressor $tRNA^{Asp}$. It is generally assumed that this position, together with residues 25 and 45, is involved in interactions maintaining the tertiary structure of tRNAs (McClain, 1993a). In the yeast aspartic system, it was shown that the position of the D-stem as well as the conformation of the whole tRNA are in part dependent on the 25·10·45 triple interactions. In that context, the G10·U25 base pair is considered to act as a conformational identity element due to its critical role in recognition (Pütz et al., 1991) and the absence of direct contacts with AspRS (Cavarelli et al., 1993). All these observations strongly support the idea that the lysine identity acquired upon G10A mutation has to be attributed (as already assumed for the A3G mutation) to conformational changes in the tRNA due to modification of the 25·10·45 interaction network.

Mutation C38A led to the strongest suppression phenotype, with mainly lysine-inserting activity. This may be compared to the results obtained by Normanly et al. (1990), where five nucleotides had to be changed in the anticodon arm and loop in order to get an active amber $tRNA^{Asp}$ charging lysine. The lysine acceptance obtained upon C38A mutation may not rely on a structural effect because position 38 is not known to contribute significantly to the general $tRNA^{Asp}$ conformation. More likely, this mutation might give rise to new interaction possibilities with LysRS at the anticodon level, favoring, by a distant effect, the functional positioning of the acceptor helix. Whether these contact changes consist of suppression of antideterminants, creation of positive identity elements, or both, cannot be decided. However, mutation C38A clearly changes an important identity element of $tRNA^{Asp}$ (Nameki et al., 1992), rendering the anticodon loop of the amber $tRNA^{Asp}$ identical to that of the amber $tRNA^{Lys}$ (the latter being recognized by LysRS). This would support the idea that A38 is playing a role in LysRS recognition. As for the higher suppression level, one cannot exclude that A38 changes the efficiency of this mutated tRNA in the translation process. In fact, Yarus (1982) has shown that position 38 plays a crucial role during the ribosomal step of tRNA, the preferred nucleotide at that position being an adenine.

The mutant ΔC56 has the lowest suppression efficiency and displays exclusive glutamine acceptance. Disruption of the 19·56 tertiary interaction obtained upon ΔC56 deletion obviously favors a tRNA conformation recognized by GlnRS. Glutamine identity may appear surprising a priori because mutant ΔC56 has a strong base pair at position 1·72, whereas Jahn et al. (1991) have shown that a weak interaction is required at that position for GlnRS recognition. This permits disruption of the 1·72 base pair upon interaction with

GlnRS, thereby allowing folding back of the acceptor end toward the active site of the enzyme. From these considerations, it may be concluded that G1·C72 is a weakly interacting base pair in the $tRNA^{Asp}_{CUA}$ nucleotide context. This weakening effect most likely may be due to the nucleotide at position 73; from previous work, it is known that the discriminator base influences the structure of the acceptor helix and particularly the stability of the first base pair onto which it is stacked (Lee et al., 1993). The importance of this effect was shown to be dependent on the type of nucleotides present at positions 1·72 and 73. That G73 associated with G1·C72 is, as was assumed, favoring dissociation of the first base pair, may be supported by previous results. $tRNA^{Ser1-2}$, as well as *supF* $tRNA^{Tyr}$G73, which both present the previous nucleotide combination, were shown to be misacylated by GlnRS. Lastly, what happens to the first and second base pair also depends on the whole tRNA, the protein, and the way the protein interacts with the tRNA (Lee et al., 1993). As for the low suppression activity of the ΔC56 mutant, it remains to be established whether it was due to the low charging activity of the mutant $tRNA^{Asp}_{CUA}$, or to its poor efficiency in the translation step, or both.

TABLE 2. Identities and suppression efficiency increase after phenotype improvement of two suppressors by a second mutation-selection round.[a]

tRNA mutation	Increase of suppression efficiency (%)	Amino acid acceptance	Colonies' phenotype
Initial A3G	0	Ala 74%, Lys 26%	Arg+
A3G G15A	74	Ala 80%, Lys 20%	Arg+ Lac+
A3G C17U	45	Ala 65%, Lys 35%	Arg+ Lac+
A3G U20A	30	Ala 70%, Lys 30%	Arg+ Lac+
A3G U21C	15	nd[b]	Arg+ Lac+
Initial G10A	0	Lys	Arg+
G10A A3G	171	Ala 95%, Lys 5%	Arg+ Lac+
G10A A3G U16C	273	Ala	Arg+ Lac+
G10A A3G U16A	86	Ala	Arg+ Lac+
G10A A3G U20A	69	Arg	Arg+ Lac+
G10A +C24b	10	Lys 60%, Ala 40%	Arg+ Lac±
G10A U69C	37	Lys 50%, Ala 50%	Arg+ Lac±
G10A G73U	4	Lys 66%, Ala 34%	Arg+ Lac±

[a] Suppression efficiency increases are expressed relative to the efficiencies of the parental A3G and G10A mutants (see Table 1 for these values). The increase of suppression efficiencies are indicated relative to the A3G and G10A mutants activities (see Table 1 for the absolute value). Accuracy of the measurements is within ±10%.

[b] For unexplained reasons, we couldn't isolate the DHFR protein from mutant A3G U21C.

Improvement of suppressor efficiency

The previous genetic selection permitted the isolation of $tRNA^{Asp}$ amber suppressors presenting various amino acid identities, each one resulting from mutations at different positions in the molecule (Table 2). However, as observed, suppression efficiencies remained rather low. This could be explained by the fact that (1) only part of the recognition set corresponding to the new specificity was present in the mutants, (2) $tRNA^{Asp}_{CUA}$ mutants still contained some antideterminants directed against the noncognate aaRS, or (3) that the tRNA scaffold was not optimal for enzyme recognition. Whatever the reason, it appeared that obtaining tRNAs having higher suppressing activity would require more than one mutation. The probability of isolating such multimutants from our library was low because statistically it contained only one mutation per 100 bases. Therefore, further mutagenesis and screening was done to select for more efficient suppressors (blue colonies [Arg^+, Lac^+]). Two libraries were constructed, each starting from an isolated suppressor tRNA, giving a white phenotype [Arg^+]. Mutant C38A was not retained for library construction because it already conferred a blue phenotype; as for mutant ΔC56, it was excluded, due to its very low growth rate (this mutation affects one of the conserved tertiary interaction common to all tRNAs). Plasmid DNAs corresponding to mutants A3G and G10A were chosen as candidates, subjected to a new round of PCR amplification in mutagenic conditions, and PCR products cloned in the same vector. The resulting A3G and G10A libraries were screened for suppressors as described before. Results are summarized in Table 2.

Four double mutants were isolated from the A3G library: A3G G15A, A3G C17U, A3G U20A, and A3G U21C (Fig. 3). From the G10A library, the double mutant G10A A3G was selected frequently and, more rarely, the triple mutants, G10A A3G U16A, G10A A3G U16C, and G10A A3G U20A (Fig. 4). All these mutant tRNAs had significantly higher suppression efficiencies than the parental $tRNA^{Asp}_{CUA}$ mutants used as templates in construction of the libraries. As predicted by their light blue phenotype, lesser suppression increase was observed for three additional mutants selected from the G10A library: G10A U69C, G10A G73U, and a variant of G10A with an additional C between position 24 and 25 called G10A +C24b (Table 2).

Each mutant derived from the A3G library shows alanine and lysine acceptor activities in a ratio close to the one observed for the parental A3G mutant. The four selected mutations were located in the D loop, one of them affecting the Levitt base pair 15·48, the other one substituting residue C17, as well as position 20, which, in $tRNA^{Ala}$, belongs to its identity set (G20) (McClain et al., 1991; Tamura et al., 1991) and the adjacent residue at position 21. The higher suppressor activities acquired by these mutants may be explained by several factors: (1) an increase of the in vivo aminoacylation level due to the presence of additional identity elements or better structural adjustment of the mutated $tRNA^{Asp}_{CUA}$ to the noncognate aaRS; (2) higher

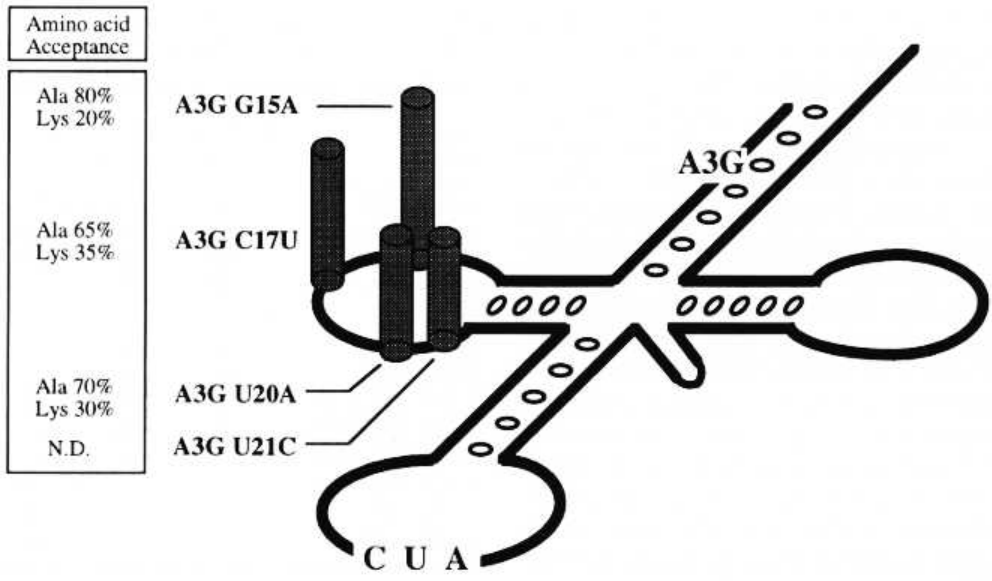

FIGURE 3. $tRNA^{Asp}$ amber suppressors selected from a library obtained by random mutation of the A3G $tRNA^{Asp}_{CUA}$ mutant. Other specifications are as for Figure 1.

translation efficiency (Yarus, 1982); or finally, (3) improved resistance against RNAse degradation. Preference should be given to the two last hypotheses because, in these cases, as observed for the previous mutants, no significant variation of the lysine/alanine ratio would be expected.

The seven mutants selected starting from the G10A (Fig. 4) library can be classified in two groups according to their suppression efficiencies. The first one contains the four most efficient suppressors: the double mutant G10A A3G and its two derived triple mutants G10A A3G U16A or C and G10A A3G U20A. The second group contains three double mutants presenting only slightly higher suppression efficiencies compared to the parental mutant G10A, but displaying a more or less important additional identity.

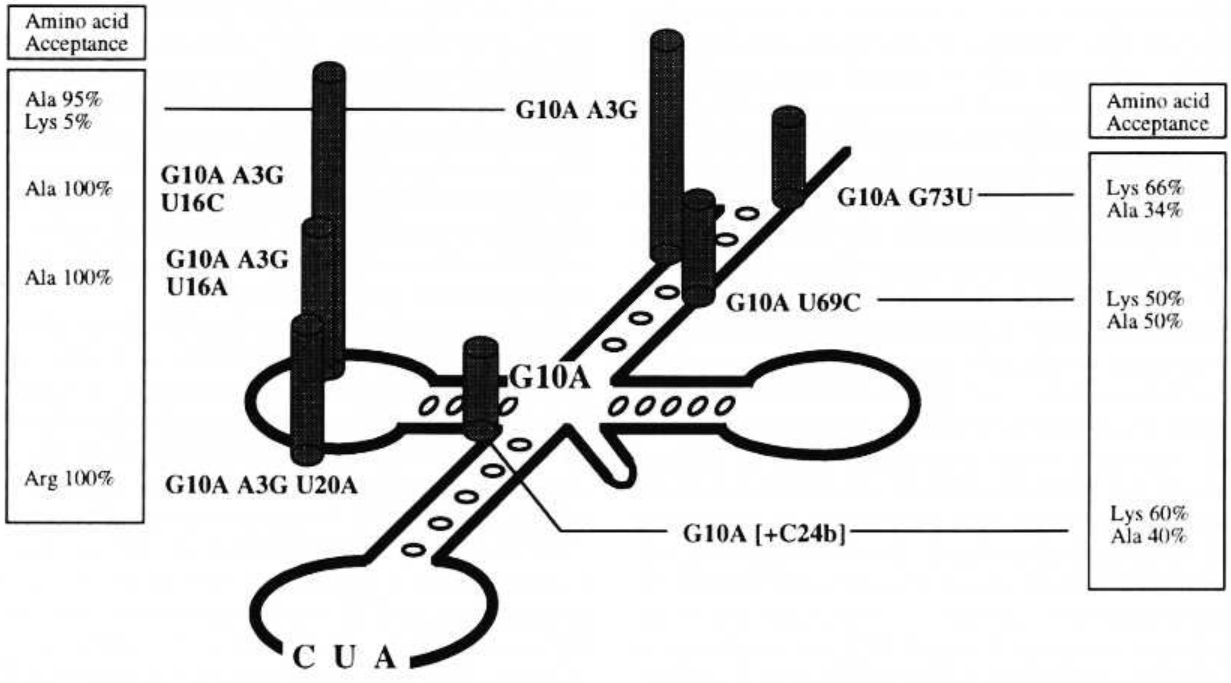

FIGURE 4. $tRNA^{Asp}$ amber suppressors isolated from a library obtained by randomly mutating the G10A $tRNA^{Asp}_{CUA}$ mutant. Other specifications are as for Figure 1.

Determination of the tRNA acceptor identities revealed that mutants of the first group were almost exclusively alanine-inserting suppressors, except for the triple mutant G10A A3G U20A, which only inserted arginine. An interesting point is that, when G10A is associated with A3G, it does not induce new identities, but only improves the alanine suppressor activity already displayed by the single A3G mutant. This observation reinforces the previous hypothesis, according to which the G10A effect is thought to consist of changes in tertiary structure constraints that would confer more flexibility to the tRNA conformation, thereby favoring a better adaptability of the tRNA acceptor arm to the aaRS active center.

The alanine suppressor activity of the double mutant G10A A3G is further enhanced by changing residue U16 into A16 or C16. This last mutant is the most active one, with a fourfold increase relative to the parental G10A mutant. This may be ascribed to the fact that $tRNA^{Ala}$ contains a cytosine at position 16, the latter belonging to its variable pocket, which contains one of the identity elements of this tRNA, the G20 (McClain et al., 1991). However, we cannot exclude the possibility that C16 is also an identity element of $tRNA^{Ala}$ and/or that residue U16 is playing a moderate antideterminant role against recognition by AlaRS in the amber suppressor $tRNA^{Asp}$ context.

Mutant A3G G10A U20A is of particular interest because it inserts exclusively arginine, whereas the related A3G U20A mutant quite predominantly presents alanine acceptance. The arginine specificity of the triple mutant clearly has to be attributed to the presence of A20, which, together with C35, was shown to be an essential identity element of $tRNA^{Arg}$ (McClain & Foss, 1988b; McClain et al., 1990; Tamura et al., 1992). However, as shown by considering A3G U20A and A3G U20A G10A mutants, A20 only specifies arginine identity in vivo when associated with G10A. Clearly, structural modifications induced by changing the G10·U25 wobble pair into a classic Watson-Crick base pair upon G10A mutation cause A20 to productively interact with ArgRS, the resulting mutant being a more efficient substrate for ArgRS than AlaRS. That A3G is also contributing to this structural requirement is more than likely because no G10A U20A mutant was isolated from the library.

Concluding remarks

Analysis of the different suppressors reveals that acylation activity of $tRNA^{Asp}_{CUA}$ and specificity is triggered by mutations either creating new identity elements or affecting the structural properties of the tRNA in a way that promotes the functional presentation of identity elements already present in $tRNA^{Asp}_{CUA}$. The G10A mutation is thought to induce such changes (probably conferring more flexibility to the tRNA conformation), thereby revealing the recognition potentialities of $tRNA^{Asp}_{CUA}$. When alone, the G10A mutation exclusively confers lysine acceptance, thus indicating that the amber suppressor $tRNA^{Asp}$ has a subjacent lysine identity. When G10A is associated with the alanine identity element G3·U70 by the A3G mutation, alanine acceptance prevails over lysine. If the suppressor contains both the alanine G3·U70 and the arginine A20 identity elements, the G10A $tRNA^{Asp}_{CUA}$ mutant behaves as an exclusive substrate for ArgRS. Noticeably, this arginine identity character is much more pronounced in yeast $tRNA^{Asp}$, because it is significantly aminoacylated in vitro by the homologous ArgRS (Gangloff & Dirheimer, 1973) in contrast to the *E. coli* $tRNA^{Asp}$ (Tamura et al., 1992); this implies that in yeast, competition between AspRS and ArgRS plays a major part in determining the specificity of in vivo $tRNA^{Asp}$ aminoacylation.

The highest suppression activity obtained remained relatively low; it corresponded to less than 1% of the β-galactosidase activity of the control strain. Efforts to select suppressors having higher activities remained unsuccessful. Either more than three changes are necessary to get suppressors having higher efficiencies, or the corresponding mutants are not present in the library we screened; indeed, although PCR amplification in mutagenic conditions is known as a highly efficient method to mutagenize a targeted DNA region, it does not allow one to obtain a homogeneous library statistically mutated at each position. For example, the mutant G10A A3G was frequently isolated from the G10A library, whereas it was not obtained starting from the A3G library. This could mean that Taq polymerase fidelity, in the presence of Mn^{2+} is influenced by the primary sequence context of the template DNA.

Last, we failed to obtain a $tRNA^{Asp}$ suppressor displaying aspartic acid acceptance. Evidently, modification of the $tRNA^{Asp}$ anticodon into an amber triplet impairs the functionality of its identity map in such a way that only extensive mutational changes may compensate for loss of aspartic acid acceptance. This is consistent with previous work showing that position 34 of *E. coli* $tRNA^{Asp}$ is a strong identity element, its substitution resulting in a 3,200-fold decrease of specificity (Nameki et al., 1992). This effect is too important to be attributed to the simple loss of the binding energy between AspRS and G34 because, in the yeast aspartic system where the same G34-AspRS interaction is present, disruption of this bond resulted in only a 66-fold specificity decrease (Martin et al., 1993). It is most likely that the *E. coli* enzyme, upon interacting with G34, also triggers (by a distant effect) functional positioning of the acceptor stem in the transition state of acylation catalysis. Lastly, it cannot be excluded that C34 is also acting as an antideterminant against AspRS recognition. Taken together, these results strengthen the idea that isolation of $tRNA^{Asp}$ amber suppressors

would require important structural modifications to compensate for perturbations induced by the anticodon change, this without impairing the structural elements necessary for interaction with the translational machinery components. Such a challenge may reasonably be taken up using a bacterial strain containing a reporter protein having an amber codon at the place of an essential aspartic acid residue.

MATERIALS AND METHODS

Chemical reagents, enzymes, nucleic acids, and standard procedures

Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Biosynth AG and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was from BRL. Restriction enzymes, T4 DNA ligase, Taq DNA polymerase, and T4 polynucleotide kinase were purchased from Boehringer-Mannheim. T7 DNA polymerase was from Pharmacia. All enzymes were used according to the manufacturer's instructions. [α^{35}S] dATP was from Amersham. Standard procedures were used for plasmid preparations and DNA manipulations.

Bacterial *E. coli* strains and vectors

The *E. coli* indicator strain 121R [*F'(pro⁺, lacI-Z$_{am}$181),ara, argE$_{am}$, Δ(lac-pro), nalA, rpoB, thi, recA, srl300::Tn10*] (Miller & Albertini, 1983) was used for the selection of active amber tRNA^{Asp} genes and for β-galactosidase activity measurements (provided by Dr. M. Springer).

The amber tRNA^{Asp} gene was constructed by oligonucleotide assembling and cloned into pTrc99-B according to Martin et al. (1993). The tRNA was overexpressed using the strong promoter *trc* with IPTG induction (Aman et al., 1988).

The pDA5YC (a generous gift from Dr. Choll W. Kim, UCLA) was used to determine the tRNA acceptor specificity. It carries the dihydrofolate reductase gene (*fol$_{am}$*) placed under the control of an IPTG-inducible promoter.

The strain UF261 [121R (*lacI-Z⁺*)] was used as a control for β-galactosidase measurements.

Construction of a randomly mutated library of amber tRNA^{Asp}

The randomly mutated library of amber tRNA^{Asp} was constructed using a modified PCR (Leung et al., 1989). The tRNA$^{Asp}_{CUA}$ synthetic gene was amplified using two oligonucleotides complementary to upstream and downstream sequences. The reaction mixture was essentially the same as described previously (Martin et al., 1995); the presence of 0.5 mM $MnCl_2$ in the mixture favors Taq polymerase errors, up to a level of 1 error per 100 bases polymerized (Leung et al., 1989). The polymerization products were then ligated into the *Eco*R I and *Bam*H I sites of pTrc99-B and the ligation mixture was used to transform strain 121R. The frequency of mutation was controlled by direct sequencing of several amber tRNA^{Asp} genes.

Selection of active amber tRNA^{Asp}

The library of mutated amber tRNA^{Asp} genes was introduced into strain 121R by transformation and plated on LB-ampicillin. After 14–36 h incubation at 37 °C, colonies were replicated onto M9 medium supplemented with ampicillin (100 μg/mL), IPTG (1 mM), and the chromogenic substrate X-gal (20 μg/mL). After 24–72 h of incubation at 37 °C, small white colonies [Arg⁺] and normal-sized blue colonies [Arg⁺, Lac⁺] appeared on M9 medium. To determine whether the phenotype was linked to the amber tRNA gene, plasmid DNA was prepared from the [Arg⁺] colonies and used to retransform the indicator strain 121R under the same selective conditions. Plasmids containing the amber tRNA^{Asp} genes were purified and the tRNA^{Asp} gene sequenced using an appropriate primer.

Quantitative determination of the suppression efficiencies

The suppression efficiencies of the different tRNA$^{Asp}_{CUA}$ mutants were determined by quantitative measurement of the β-galactosidase activities resulting from the suppression of an amber mutation in the *lacI-Z* fusion. Activities were normalized to the β-galactosidase activity of a standard strain UF261; activities were measured using the LumiGAL kit (Clontech, California).

Determination of the amber tRNA^{Asp} identities by the DHFR suppression assay

The amino acid acceptance of a selected mutant tRNA$^{Asp}_{CUA}$ was determined by a procedure based on the suppression by this tRNA of an amber mutation located in the DHFR gene *fol*. This occurs in strain 121R co-transformed with both pTrc99-B containing the mutated genes of tRNA$^{Asp}_{CUA}$ and by plasmid pDA5YC, which contains the *fol$_{am}$* gene. DHFR expression was induced by IPTG and the protein purified to near homogeneity by one-step affinity chromatography on an agarose-methotrexate column as described previously (Normanly et al., 1986). Finally, the NH_2-termini of the purified DHFR was sequenced on an automatic protein analyzer (Applied Biosystem 470 A) up to the 12th residue. Starting from 40 pmol of DHFR, a given amino acid may be detected unambiguously at position 10 when present at a level of 2%.

ACKNOWLEDGMENTS

We thank M.L. Gangloff for technical assistance, A. Hoeft for DNA oligonucleotide synthesis, and C. Lichté for N-terminal protein analysis. We are indebted to Dr. M. Springer for the gift of the strain 121R. We are grateful to Barbara Windsor and Joseph Ng for critically reading this manuscript. This work was supported by the Centre National de la Recherche Scientifique, Ministère de la Recherche et de l'Enseignement Supérieur (MRES) and the Association pour la Recherche sur le Cancer (ARC). F.M. was supported by a fellowship from the MRES.

Received May 15, 1996; returned for revision June 14, 1996; revised manuscript received June 26, 1996

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