

Genetic selection for active *E.coli* amber tRNA^{Asn} exclusively led to glutamine inserting suppressors

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ABSTRACT

Suppressor tRNAs are useful tools for determining identity elements which define recognition of tRNAs *in vivo* by their cognate aminoacyl-tRNA synthetases. This study was aimed at the isolation of active amber tRNA^{Asn}. Nineteen mutated tRNA^{Asn}_{CUA} having amber suppressor activity were selected by an *in vivo* genetic screen, and all exclusively inserted glutamine. From analysis of the different mutations it is concluded that glutamine accepting activity was obtained upon reducing the interaction strength between the first base pair of the tRNA^{Asn}_{CUA} by direct or indirect effects. Failure to isolate tRNA^{Asn}_{CUA} suppressors charged with asparagine as well as other evolutionary related amino acids is discussed.

INTRODUCTION

The accurate conversion of genetic information into amino acid sequence is mediated by tRNAs which relate a given codon triplet to the corresponding amino acid. This requires high specificity from the tRNAs both in codon pairing as well as in amino acid acceptance. The high specificity of the aminoacylation step raises the question as to how aminoacyl-tRNA synthetases (aaRS) discriminate between cognate and non-cognate tRNAs, considering that these molecules all appear to share a certain degree of structural similarity, presumably because they have to interact with common molecules of the protein synthesis machinery. The important work devoted to elucidate this problem shows that, within the extensive regions interacting with the cognate enzyme, each tRNA is recognised by a unique combination of only a few nucleotides known as identity elements (reviewed in 1–5); they are composed of positive elements (determinants) leading to productive interactions with the cognate synthetase and negative elements (anti-determinants) which prevent the tRNA from mis-recognition by the other 19 aminoacyl-tRNA synthetases. The main identity nucleotides are generally found within the amino acid acceptor helix and the anticodon loop with the exception of tRNAs specific for serine, histidine, leucine and alanine which are not recognised by their anticodon (3,5). At position 73 is located the so-called discriminator base whose contribution as an identity element has been established for many tRNAs. However, a specific role in

identity may not be assigned to this nucleotide (6). It was shown to contribute to the conformation of the acceptor arm (7–8) and has been proposed to stabilise the transition state of the acylation step (9–10) upon interaction with aaRS. In some cases, nucleotide 73 may also act as an anti-determinant, preventing productive interactions with non-cognate aaRSs. Identity also depends on determinants located within the variable pocket; these either directly interact with the enzyme or confer to the tRNA the appropriate conformation required for optimal presentation of the determinant elements so that they can properly contact the aaRS (3).

Identity elements of tRNAs may be explored by several types of *in vitro* and *in vivo* approaches. The *in vitro* methodology is based on acylation analysis of tRNA transcript variants produced by run-off transcription of the corresponding synthetic mutated tRNA genes. The main limitation of this method comes from the facts that: (i) tRNA transcripts have no modified nucleosides, whereas they may function as determinants (11–12) or anti-determinants (13) and (ii) the effects of mutations on aminoacylation are tested in the absence of non-cognate aminoacyl-tRNA synthetases so that only recognition sites may be detected. These limitations are overcome by the *in vivo* methodology developed by Normanly *et al.* (14): mutated nonsense suppressor tRNAs are expressed on a plasmid in bacterial cells. The identity of the charged amino acid is determined after purification and sequencing of dihydrofolate reductase protein (DHFR), the gene which contained an amber triplet at position 30 from the translation start site. Obviously, the *in vivo* approach cannot be used for tRNAs which are inactivated upon changing their anticodon in a nonsense triplet as for tRNA^{Pro}, tRNA^{Thr1}, tRNA^{Asp} and tRNA^{Asn} (15). However, with the exception of tRNA^{Asn}, activities of those tRNAs having an amber anticodon could be restored by mutations in the anticodon stem and loop (15–16). In the absence of an active tRNA^{Asn} amber suppressor, Li *et al.* (17) tried to define tRNA^{Asn} identity by an alternative method whereby they showed the importance of discriminator and anticodon bases for aminoacylation. Nevertheless it remains that delineation of identity elements including anti-determinants may only be approached efficiently by *in vivo* suppressor methodology. Here we describe selection of tRNA^{Asn} amber suppressors from a library of randomly mutated tRNA^{Asn} having an amber anticodon (tRNA^{Asn}_{CUA}) by a genetic screen. The aminoacylation specificity of active isolated tRNA mutants

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was determined both by *in vivo* selective complementation and by DHFR sequencing.

Nineteen different tRNA^{Asn}_{CUA} mutants were isolated, all of which exclusively exhibited glutamine acceptance. Mutations were analysed to try to find out the way in which they functionally compensated the charging deficiency of tRNA^{Asn}_{CUA}.

MATERIALS AND METHODS

Bacterial *Escherichia coli* strains and plasmids

The *E. coli* strain 121R [*ara*, *argE*_{am53}, Δ (*lac-proB*), *nalA*, *rpoB*, *thi*, *recA56n*, *srl300::Tn10* F'(proA⁺B⁺, *lacI-Zam181*)] (18) was used as an indicator strain for amber tRNA^{Asn} suppression assays (provided by Dr M. Springer).

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

The strain BT52 [F' *lacZ1000*_{am}, *trp*_{am}, *T6*_{am}, *BF23*_{am} *str*⁻, *su*⁰, λ ^s] provided by Dr Inokuchi (19) and KL986 [F'(*trpA*_{am234}), *argH*, *glyV55*, Δ (*tonB-trpAE1*)] provided by Dr Hijazi (20) were used for the selective complementation assays.

The wild type and mutated tRNA^{Asn}_{CUA} genes were cloned into the vector pTrc99-B according to Martin *et al.* (22) and Kleina *et al.* (16). It contains the strong promoter *trc*, a hybrid of promoters *lac* and *trp*, upstream of a multiple-cloning-site followed by two transcription termination signals (T1 and T2) of the ribosomal operon *rrnB*. In addition, this plasmid carries the *lacI*^q gene encoding the lac repressor allowing efficient repression in the absence of IPTG (isopropyl- β , D-thiogalactopyranoside) induction.

The pDA5YC vector (a generous gift from Dr Choll W. Kim, UCLA) was used to overproduce DHFR from the *fol*_{am} gene placed under the control of an IPTG inducible promoter.

Enzymes, chemicals, nucleic acids and standard procedures

Restriction enzymes, T4 DNA ligase, *Taq* DNA polymerase and phage T4 polynucleotide kinase were obtained from Boehringer Mannheim. T7 DNA polymerase was obtained from Pharmacia. All enzymes were used according to the manufacturer's instructions. [α -³⁵S]dATP was obtained from Amersham. Standard procedures were used for plasmid preparation and DNA manipulations (22). IPTG and 5-bromo-4-chloro-3-indoyl- β -galactopyranoside (X-gal) were from BRL.

Construction of a randomly mutated library of tRNA^{Asn}_{CUA}

The randomly mutated library was obtained by PCR amplification of the tRNA^{Asn}_{CUA} synthetic gene in the presence of manganese: it was shown that this cation decreases the *Taq* DNA polymerase fidelity leading to a high frequency of mutation (23). The PCR conditions were adjusted to 0.5 mM MnCl₂ in order to obtain on average one error per 100 nucleotides polymerized. In addition, the PCR reaction mix contained 100 pmol of two synthetic oligonucleotides respectively complementary to the upstream and downstream region of the synthetic tRNA^{Asn}_{CUA} gene, 100 ng of the recombinant plasmid pTrc99-B with the synthetic gene of the tRNA^{Asn}_{CUA}, 10 mM β -mercaptoethanol, 10% (v/v) of DMSO (dimethyl sulfoxide), 0.5 mM MnCl₂, 1 mM of each of the four deoxynucleotides (dATP, dCTP, dGTP, dTTP),

0.166 M (NH₄)₂SO₄, 0.67 M Tris-HCl (pH 8.8), 61 mM MgCl₂, 67 mM EDTA (pH 8) and 1 U *Taq* DNA polymerase. The PCR products were digested with *Eco*RI and *Pst*I for 16 h and religated in 0.1 pmol of pTrc99-B previously cut with *Eco*RI and *Pst*I and subsequently dephosphorylated. This ligation mixture was used to transform the strain 121R. The mutation frequency was estimated by sequencing 20 plasmids chosen at random from the mutated amber tRNA^{Asn}_{CUA} gene library (24).

Selection of tRNA^{Asn}_{CUA} suppressors

The library of mutated tRNA^{Asn}_{CUA} genes was transformed in strain 121R and plated on M9 medium supplemented with ampicillin, IPTG and X-Gal. After incubation at 37°C for 1–3 days, two kinds of clones were observed: blue colonies with a quite normal growth rate and small white colonies. To confirm that the phenotypes were dependent on recombinant plasmids, the latter were retransformed into strain 121R and cultured under the same selective conditions. The plasmid with genes resulting in tRNA^{Asn}_{CUA} suppressor activity were sequenced.

Analysis of tRNA^{Asn}_{CUA} suppressors

Determination of suppressor efficiency. The suppressing efficiencies of the mutated tRNA^{Asn}_{CUA} isolated in the screen were determined as described by Miller (25), by measuring the β -galactosidase activity of strain 121R when expressing the selected recombinant plasmids. The suppressor efficiency is defined as the β -galactosidase activity of a mutant relative to the β -galactosidase activity of the standard strain UF261.

Determination of the identity of the tRNA^{Asn}_{CUA} suppressors.

(i) Amino acid-specific suppression: the determination of the tRNA identity of the mutated tRNAs was first approached by selective complementation tests using the strains BT52 and KL986. The strain BT52 contains *lacZ1000*_{am}; the [lac⁺] phenotype is obtained by suppression of the amber codon exclusively by glutamine or tryptophan. The presence of β -galactosidase activity was detected as mentioned before. The strain KL986 contains an amber mutation in *trpA*. As shown by Murgola and Hijazi (20), the phenotype [trp⁻] is reversed by an amber suppressor tRNA inserting alanine or glycine. The transformed cells were selected for by growth on glucose-M9 medium supplemented with arginine (20 mg/ml) and in the presence of ampicillin. (ii) DHFR suppression assay: the DHFR reporter protein was used to determine the type of amino acid charged on the suppressor tRNA^{Asn}_{CUA}. Strain 121R was transformed with both pTrc99-B containing the mutated tRNA^{Asn}_{CUA} suppressor genes and by pDA5YC which contains the *fol*_{am} gene. The DHFR protein was overproduced by IPTG induction and purified to nearly homogeneity in one step by affinity chromatography on an agarose-methotrexate column (14). The N-termini of the purified DHFRs were sequenced on an automatic protein analyser (Applied Biosystems 470 A) up to residue 12 from the N-terminus. Under the experimental conditions used (40 pmol of DHFR) a given amino acid is unambiguously detected at position 10 up to a level of 2%.

RESULTS

Colonies obtained from screening in the 121R strain were of two phenotypes: small white colonies (35%) and at a higher frequency

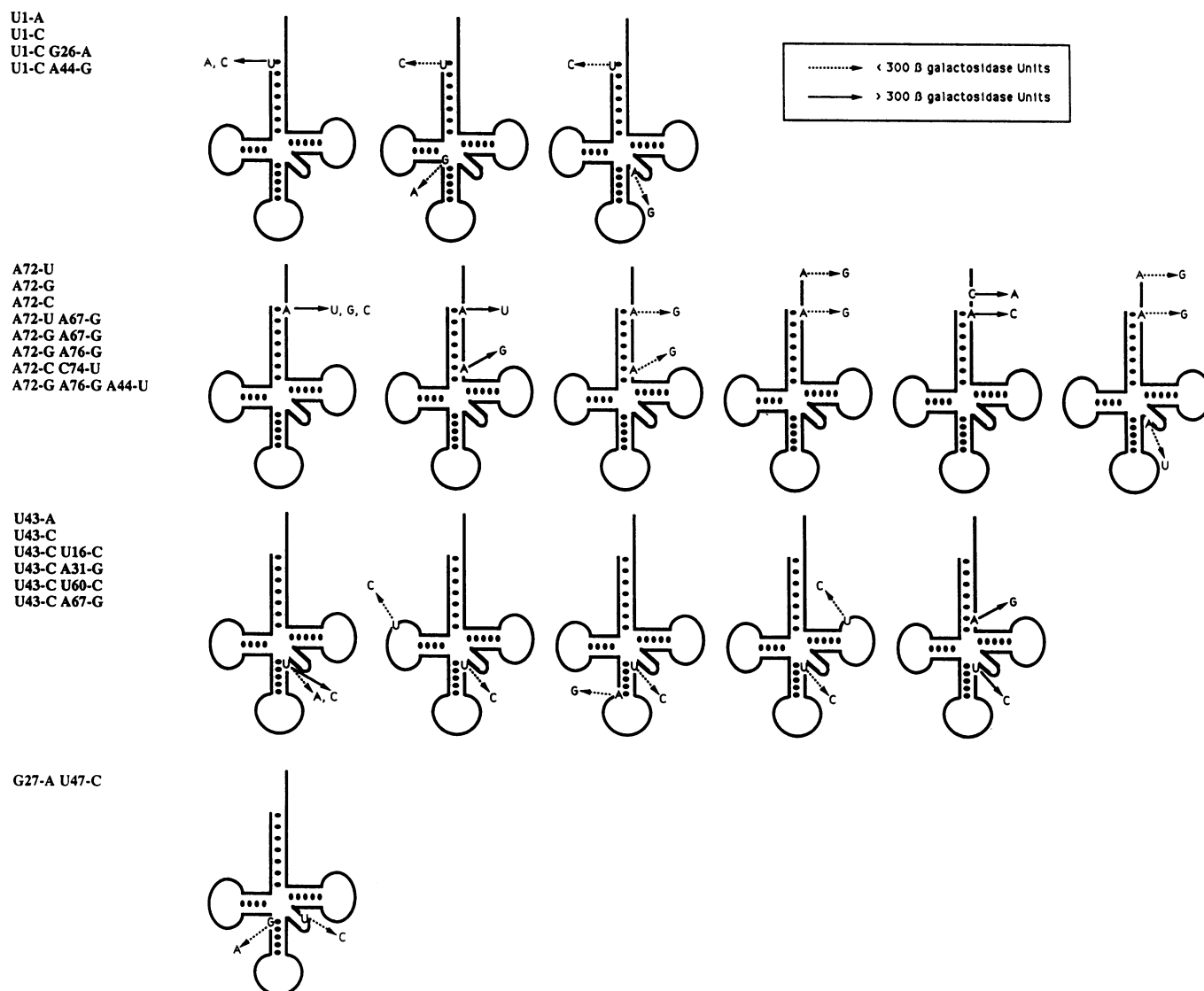


Figure 1. tRNA^{Asn} amber suppressors isolated by *in vivo* selection. Construction and screening of the randomly mutagenised plasmid library of *E. coli* tRNA^{Asn}_{CUA} genes was performed as described in the text. Arrows with full or dotted lines respectively correspond to mutations conferring suppressing efficiencies above and below 300 U β -galactosidase (see Fig. 2).

(65%), large blue colonies. Out of 63 colonies analysed, we isolated 19 different tRNA^{Asn}_{CUA} mutants (see Fig. 1) having amber suppressor activity, those containing one mutation being obtained in several exemplars. With the exception of the double mutant (G27-A, U47-C), it appears that all these *E. coli* tRNA^{Asn}_{CUA} mutants contained a mutation at one of two hot spots: the first base pair of the acceptor stem and position U43.

The blue colonies all contained only single mutations located at the first base pair of the acceptor stem (positions 1 and 72). In all cases these mutations weakened the interaction strength of this base pair: nucleotide U1 was changed into C or A so that the first base pair was C1:A72 or A1:A72. Residue A72 was substituted by U, G or C resulting in the U1:U72, U1:G72 and U1:C72 base pairs respectively.

The small white colonies contained tRNA^{Asn}_{CUA} with mutations at the first base pair as well as in other parts of the molecule: (A72-U, A67-G); (A72-G, A67-G); (A72-G, A76-G) (A72-C,

C74-U); (A72-G, A76-G, A44-U); (U1-C, G26-A) and (U1-C, A44-G). Small colonies also harboured tRNA^{Asn}_{CUA} with single mutations at position 43 (U43-C), (U43-A) and with double mutations (U43-C, A67-G); (U43-C, A31-G); (U43-C, U16-C) and (U43-C, U60-C). Finally we isolated the double mutant (U47-C, G27-A) which does not fit into either of the two preceding categories.

From suppressor activity measurements of the different tRNA^{Asn}_{CUA} mutants (Fig. 2) it appeared that the single mutant (U1-A) was the most efficient suppressor (800 U); it exhibited about 5% of the wild type activity (the β -galactosidase activity of the standard strain UF261). The other single mutants showed activities ranging from 300 to 800 U. In the case of the double or triple mutants having a mutation at the first base pair of the acceptor stem, it was observed that the second and third mutation in the tRNA generally diminished suppressor efficiency; the sole exception was the double mutant (A72-U, A67-G) where the

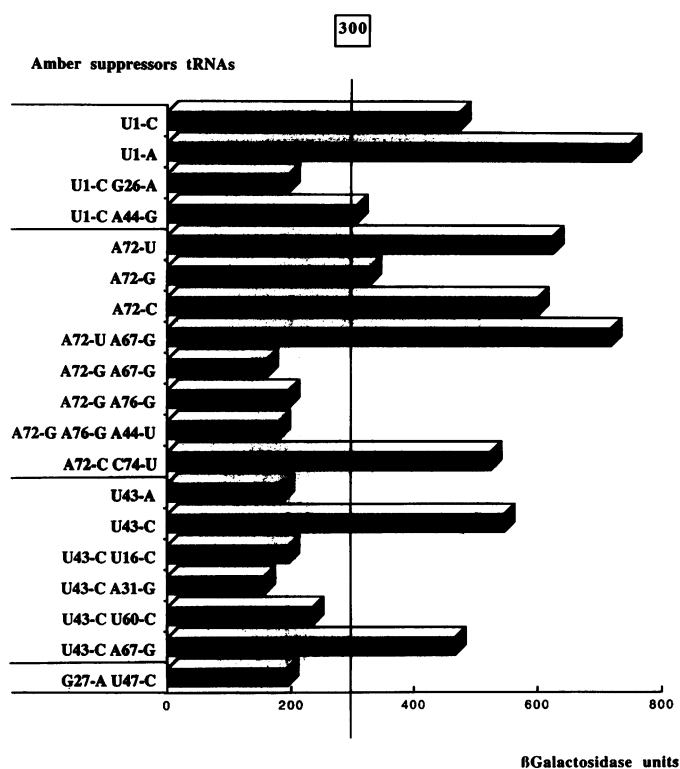


Figure 2. Histogram representing the amber suppressor efficiencies of the selected $\text{tRNA}^{\text{Asn}}_{\text{CUA}}$ mutants. The suppressor activities are evaluated in β -galactosidase units, determined according to Miller (25), they are expressed relative to the activity of the standard strain UF261. The represented activity levels are mean values calculated from three independent measurement sets. The observed standard deviations remained within 10%. If classifying the different mutants according to their respective activity levels, the same order was obtained in all three measurement sets.

β -galactosidase activity was slightly higher compared to the single mutant (A72-U).

Mutations at position 43 presented different levels of activities: the best suppressor was the single mutant (U43-C) whereas the single mutant (U43-A) only exhibited activity comparable to that of the double mutants (U43-C, U60-C), (U43-C, U16-C) and (U43-C, A31-G). Interestingly, the (U43-C, A67-G) mutant was almost as active as the single mutant (U43-C). As with mutant (A72-U, A67-G), it was observed that addition of mutation A67-G to the single mutation U43-C did not impair the suppressor efficiency. Finally, the double mutant (G27-A, U47-C) showed a suppression level equivalent to that of the double and triple mutants, i.e. about 1% of the wild type standard activity of the UF261 strain.

Identification of the type of amino acid inserted by the *E. coli* $\text{tRNA}^{\text{Asn}}_{\text{CUA}}$ suppressors was approached in two ways: first, by testing the ability of the mutants to complement strains that harboured an amber-containing gene; the activity of the corresponding protein being strictly dependent on suppression of the nonsense codon by a specific amino acid. Two strains were used: KL986 which requires an alanine or glycine insertion for growth on minimal medium and BT52 which requires a tryptophan or glutamine insertion. None of the tRNA mutants were able to complement the KL986 indicating that they were charged *in vivo* neither by alanine nor by glycine. Only a few mutants comple-

mented the amber mutation in the $\text{lacZ1000}_{\text{am}}$ in strain BT52 (U1-C), (U1-A), (A72-U), (A72-C), (A72-U, A67-G) and (A72-C, C74-U). These mutants, which correspond to the most efficient suppressors in the β -galactosidase assay may therefore be charged by glutamine or tryptophan. The identity of the suppressor tRNAs we isolated was finally defined by determining the amino acid incorporated at the amber codon (position 10 from the N-terminal end) of the gene coding for DHFR. This analysis showed that they all incorporated glutamine exclusively in the DHFR protein.

DISCUSSION

Background

The net result of competition between all aaRS present *in vivo* for a given tRNA defines its acceptance specificity. The elements of the tRNA which determine the outcome of this competition, giving preference to a given aminoacyl-tRNA synthetase, constitute the identity set. The identity elements are either positive or negative; positive elements favour the creation of productive interactions with the cognate aaRS whereas negative elements prevent the tRNA from interacting with other aaRSs.

The identity elements of tRNAs have been particularly well studied in *E. coli* by a variety of *in vivo* and *in vitro* approaches (reviewed in 5). In the peculiar case of tRNA^{Asn} , Shimizu *et al.* (26) and Li *et al.* (17) showed that the anticodon and the discriminator base are important for aminoacylation, but a broader set of elements is thought to define its identity; this may be efficiently investigated by the *in vivo* approach mentioned before using suppressor tRNA^{Asn} . However tRNA^{Asn} with a nonsense anticodon is inactive and, up to now, attempts to convert it into an amber suppressor had remained unsuccessful (1,16). Here we report an *in vivo* selection strategy for $\text{tRNA}^{\text{Asn}}_{\text{CUA}}$ suppressors using a new genetic screen.

Positions 1, 72 and 43 are crucial mutational targets for conferring glutamine acceptance to $\text{tRNA}^{\text{Asn}}_{\text{CUA}}$

The *in vivo* selection of tRNA^{Asn} amber suppressors led us to isolate 19 different $\text{tRNA}^{\text{Asn}}_{\text{CUA}}$ mutants exhibiting suppressor activity (Fig. 1) and which all exclusively accepted glutamine. With the exception of the (G27-A, U47-C) mutant, they all were found to have mutation at one of two 'hot spot' positions, either at the first base pair of the acceptor stem or at position 43. These mutations were found either singly or associated with one or two additional modifications elsewhere in the tRNA. However, it must be emphasised that the addition of a second mutation (excepting A67-G to A72-U or U43-C) reduces suppressor activity; it is likely that in most cases these second and third mutations have been selected as they are tolerated because they only moderately impair the activity.

It is worth noting that mutations at positions 1 or 72 all resulted in decreased interaction strength between the first base pair if compared to the original U1:A72 Watson-Crick base pair. The most frequently isolated mutants (33 of the 63 analysed) had U1-C and A72-G substitutions, leading respectively to the pairings C1:A72 and U1:G72. The weakest interactions were obtained for mutants with A:A, U:U and C:U base pairs. When comparing the suppression efficiencies of all these mutants (see Fig. 1 and 2), it is clear that, the weaker the first base pair

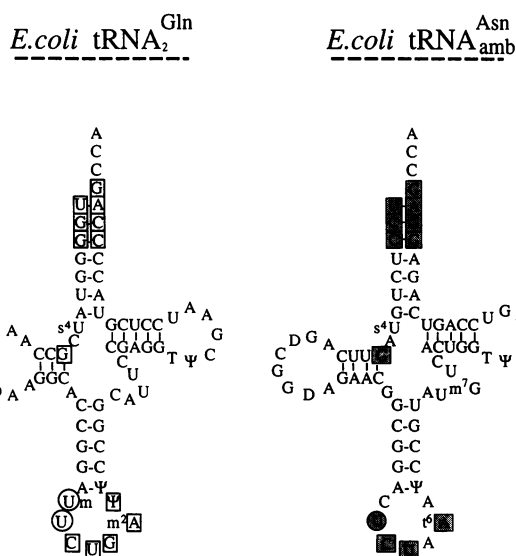


Figure 3. Cloverleaf representation of tRNA^{Asn} and tRNA^{Gln} of *Escherichia coli*. The sequence of the major tRNA^{Gln} (Gln2) is represented with boxed bases corresponding to the identified recognition elements for GlnRS (29). The identity nucleotides of tRNA^{Gln} which are found to be conserved in tRNA^{Asn}_{CUA} are shown in shadowed boxes. Circled positions may contribute to glutamine identity by stabilising the proper anticodon loop conformation.

interaction the stronger the amber suppressing activity of the mutant tRNA^{Asn}_{CUA}.

tRNA^{Asn}_{CUA} shares its main recognition elements with tRNA^{Gln}

The glutamine acceptance of mutated tRNA^{Asn}_{CUA} is not surprising in the light of what we know about tRNA^{Gln} recognition by GlnRS, from biochemical and crystallographic studies on the tRNA^{Gln}–GlnRS complex (27–29). It appears that tRNA^{Asn}_{CUA} shares most of its essential identity determinants with tRNA^{Gln} (see Fig. 3): these consist of U35, the main identity element of the anticodon which is also present in the amber anticodon, the weak U1:A72 base pair, the discriminator base G73 and both C2:G71 and C3:G70 base pairs. The U1:A72 base pair plays a major role in GlnRS discrimination for tRNAs; its breaking upon GlnRS binding is essential for activity since this allows the acceptor end to fold back into the catalytic site. As suggested by Jahn *et al.* (1991) (30), the free energy cost of breaking the terminal U1:A72 base pair may be considered as a discriminating factor in tRNA^{Gln} recognition. The G73 has a predominant function in that it stabilises this terminal hairpin conformation through hydrogen bonds with A72 (29). Base pairs C2:G71 and C3:G70 form direct and water-mediated contacts in the minor groove with GlnRS; the fact that they are in opposite presentation in tRNA^{Asn} is probably not of importance since these pairs interact in the minor groove: in that configuration G–C and C–G are thought to possess similar functional groups at more or less identical positions (31).

Glutamine acceptance activity conferred by all mutations to tRNA^{Asn}_{CUA} results from weakening of the first base pair

tRNA^{Asn}_{CUA}, despite containing several main identity elements of tRNA^{Gln}, must be additionally modified by mutation to gain glutamine accepting activity. The reason of this recognition failure is clearly revealed by the effect of mutations of the first base pair of the acceptor stem: they always resulted in a dramatic weakening of the interaction strength at that position. Thus, this substantiates the idea that tRNA^{Asn}_{CUA} does not have the appropriate structure which, upon interaction with GlnRS, would allow the activity essential disruption of position 1–72, as in tRNA^{Gln}. It must be concluded from this fact that each selected suppressor contains at least one mutation triggering disruption of the acceptor stem. In the case of mutations at position 43, this must obviously occur by an indirect effect. This could consist of a reorientation of the acceptor stem favouring the native U1–A72 disrupting interactions with GlnRS; it is well known from numerous studies that the central core of a tRNA, including position 43, is important for tRNA identity in that it determines the tertiary conformation required for optimal presentation of the tRNA identity elements to the aaRS (32). Conversely it has been shown for *E.coli* tRNA^{Ala} that modifications in this domain compensate for inactivating mutations in the acceptor stem (33). In the case of tRNA^{Tyr}, Schultz and Yarus (34–36) showed that mutations at the top of the anticodon stem (positions 27 and 43) in concert with a mutation in the D loop, allow mis-aminoacylation and facilitate abnormal near cognate pairings between the codon and the anticodon; they concluded that these positions play a primordial role in maintaining the spatial configuration of the molecule, modifications in these positions rendering the tRNA more flexible.

Mutation A67-G found in double mutants, is the sole modification which, when coupled to another mutation (A72-U, A67-G) increases the level of suppressor activity. This A67-G mutational effect may not consist of a stability decrease of the first base pair, since the latter is already completely unpaired by A72-U in the double mutant. Thus, it is possible that A67-G triggers a more functional positioning of the 3' end respective to the catalytic center. Stabilising properties of some positions in the acceptor stem have already been evident from early genetic and biochemical studies. Thus it was shown that the tyrosine-accepting specificity of amber suppressor tRNA^{Tyr} is shifted to glutamine by base substitution in the acceptor stem resulting in a weaker base pair (37-39). The sequence dissimilarity of tRNA^{Tyr} and tRNA^{Gln} acceptor stems led them to conclude that the effect was due to a change in orientation of the acceptor stem rather than to modification of the chemical nature of the mutated position.

As to the double mutant (G27-A, U47-C), its position in the variable pocket would be consistent with an effect on the configuration of the acceptor arm, favouring the wild type disrupting interactions of the U1:A72 base pair, as proposed for position 43.

But as a general remark, it has to be emphasised that the observed differences in suppressor activity levels may not simply be due to variations of acylation activities but also might reflect improved or reduced efficiencies of the mutated tRNAs in the translation step.

Finally concerning the double and triple mutants containing substitutions in the terminal CCA (A72-G, A76-G), (A72-G, A76-G, A44-U), (A72-C, C74-U), Reuven *et al.* (41) showed

that mutations at the CCA end are repaired by CCA-repairing enzymes *in vivo* (40).

Concluding remarks

The question remains of why we were not able to select tRNA^{Asn}_{CUA} accepting asparagine or amino acids corresponding to aaRSs evolutionary related to AsnRS like LysRS or AspRS (43,44).

It is clear that there is only limited chance of isolating aspartic acid charging suppressors in the light of previous studies which have shown that AspRS does not recognise the general identity map of tRNA^{Asn}; tRNA^{Asn} having a GUC anticodon (specific for aspartic acid) is not aspartylated *in vivo* (17). In addition, the fact that the tRNA^{Asn}_{CUA} anticodon differs by two positions from the one of tRNA^{Asp} further lowers the probability for shifting to aspartic acid identity by compensating mutations.

In contrast to tRNA^{Asn}_{GUC}, tRNA^{Asn}_{CUU} (CUU being specific for lysine), is acylated *in vivo* by lysine, but changing its anticodon to an amber triplet by U36→A substitution inactivates the molecule (17). Surprisingly, tRNA^{Lys} with a CUA anticodon is still lysylated *in vivo*, thus indicating that the role of A36 in recognition of tRNA^{Asn} and tRNA^{Lys} by LysRS is dependent on tRNA context.

As in the case of tRNA^{Asp}, activity of tRNA^{Asn} is abolished by replacing its anticodon by an amber triplet (tRNA^{Asn}_{CUA} differs by two anticodon nucleotides from the native tRNA^{Asn}). This may be ascribed to an affinity decrease as well as to diminution of the rate constant (k_{cat}) of the tRNA^{Asn}_{CUA} acylation reaction. The latter possibility implies that a structural change is impairing the optimal functional fit of the acceptor end in the transition state. Assuming the k_{cat} effect would be predominant, as generally observed when substituting anticodon bases in tRNAs, failure to isolate active tRNA^{Asn}_{CUA} by mutational correction points to the importance of the structural change induced by replacing the tRNA^{Asn} anticodon by an amber triplet. This might suggest that base substitution at position 34 and 36 not only suppresses positive identity elements but also creates strong anti-determinants against AsnRS recognition. Suppression of this putative anti-determinant effect could be a way to obtain charging of tRNA^{Asn}_{CUA} by asparagine. This may be achieved with AsnRS variants having lost their capacity to negatively interact with the amber anticodon.

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