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MODULATION OF THE SUPPRESSION EFFICIENCY AND AMINO ACID IDENTITY OF AN ARTIFICIAL YEAST AMBER ISOLEUCINE TRANSFER RNA IN *ESCHERICHIA COLI* BY A G-U PAIR IN THE ANTICODON STEM

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The artificial amber suppressor corresponding to the major isoleucine tRNA from yeast (pVBt5), when expressed in *E. coli*, is a poor suppressor of the amber mutation *lacI*$_{am181}$-*Z*. By analysing mutant forms, we could show that this was due to the presence of a U30-G40 wobble pair in the anticodon stem of the yeast tRNA and not to the level of the heterologously expressed tRNA. Efficient suppressors were obtained by restoring a normal U30-A40 or G30-C40 Watson-Crick pair. *In vivo* the mutant forms are exclusively charged by the bacterial lysyl-tRNA synthetase (LysRS), whereas the original yeast amber tRNA is charged at a low level by *E. coli* glutaminyl-tRNA synthetase (GlnRS) and LysRS. The inversion of the U30-G40 pair also induces a loss of the Gln identity. We conclude from these experiments that the U30-G40 base pair constitutes a negative determinant for LysRS interaction which operates either at the level of complex formation or at the catalytic step. As no direct contacts are seen between GlnRS and positions 30-40 of the complexed homologous tRNA, the U30-G40 pair of pVBt5 is believed to influence aminoacylation by GlnRS indirectly, probably at the level of the anticodon loop conformation by favouring an optimal apposition of the anticodon nucleotides with the protein.

Within a given organism, each of the present day tRNA sequence is maximized for translational efficiency and is resolved in a single amino acid specificity by a small number of nucleotides defined as the identity set for a given tRNA-synthetase. To determine the identity of a tRNA *in vivo*, Normanly et al.(1) have developed a genetic method which uses suppression of an engineered amber codon at position 10 of the *Escherichia coli* (*E. coli*) *fol* gene. In this method the amino acid that is charged on the tRNA suppressor is deduced from the N-terminal protein sequencing of the suppressed dihydrofolate reductase (DHFR). The identity of a tRNA results from positive interactions with specific nucleotides and/or indirect readout of sequence dependent tRNA conformations (2) with the cognate aminoacyl-tRNA synthetase, while these same sites (and/or others) undergo negative interactions with non-cognate tRNA synthetases. The discrimination between a productive and non-productive complex operates mainly at the level of k_{cat} during the transition state complex formation. *In vivo* there is another level of discrimination which operates at the level of complex formation. Potential productive complexes with non-cognate synthetases are prevented by the outcome of the competition between synthetases for a given tRNA. Competition

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between synthetases has clearly been shown to help discrimination in at least two homologous systems: the Gln/Tyr (3) and Ala/Tyr (4).

In a heterologous system, a given tRNA does not necessarily retain its original amino acid specificity because the nucleotide sequences of the tRNA may bear dominant determinants for a host heterologous tRNA-synthetase or avoid blocking determinants that prevent misaminoacylation in the homologous system. For example, the amber suppressor derived from the *E. coli* tyrosine tRNA, when produced in very large amounts in yeast, is aminoacylated exclusively with leucine (5) whereas mutants of the amber *E. coli* initiator tRNA are aminoacylated with tyrosine (6).

To further get an insight into the heterologous tRNA/synthetase relationship, we used the yeast major isoleucine tRNA for *in vivo* recognition studies in *E. coli*, taking advantage of the DHFR amber suppression test. A complete collection of *E. coli* amber suppressor tRNA genes corresponding to the 20 amino acids has shown that, while half of the amber suppressors tested retained the original amino acid specificity, the others were mischarged either by Gln or Lys (7). Among these, two amber tRNA alleles for isoleucine were tested and did not show the same amino acid acceptance.

Compared to the *E. coli* isoleucine tRNAs, the artificial yeast amber $tRNA^{Ile}$ species supplies sequence variations in the acceptor and in the anticodon regions that can modulate its recognition by GlnRS or LysRS.

MATERIAL AND METHODS

Strains and plasmids

E. coli 121 is *F' lacI-lacZ181am proB*$^+$/D(*lac-pro*) *argE*$_{am}$ *nalA, rpoB, thi, ara. E. coli* UF261 is identical to *E. coli* 121 except being Lac$^+$. JM101*mut*D5 is *F' traD36 proAB*$^+$*lacI*^{q} *lacZ*DM15/*supE thiDlac-proAB*. All were obtained from Dr. M. Springer (IBPC, Paris). The plasmid pDa5YC was a gift from Dr. Choll W. Kim (UCLA). Plasmid pMEX8 from MoBiTec GmbH, Göttingen, RFA, contains a *ptac* promoter on the 5' side of the *Eco*RI restriction site and a rrnC terminator sequence on the 3' side of the *Pst*I restriction site.

Design and cloning of tRNA genes

The gene for the yeast $tRNA^{Ile}$ amber suppressor was constructed from two complementary synthetic oligonucleotides flanked by *EcoR*I and *Pst*I sites and inserted in the polylinker region of pMEX8. Its sequence was verified using the dideoxy method of Sanger (9). The single-stranded DNA phage production was done as described by Viera and Messing (10). Synthetic mutants of pVBt5 amber suppressor were constructed in a similar way.

In vivo random mutagenesis and selection of tRNA amber suppressor mutants

The *E. coli* JM101*mut*D5 strain was infected with pVBt5 phagemid. After 52 h of growth, helperphage M13K07 was added and the mutated single-stranded DNA phages were collected and used to transfect the *E. coli* 121 strain. This strain has no ß-galactosidase activity, but bears a fused *lacI*$_{amb}$-Z gene on its episome. Any suppression of the amber stop codon in the *lac*I part of the fused *lacI*$_{amb}$-Z gene leads to an active ß-galactosidase (11). 10^8 infected *E. coli* cells were plated on minimal medium containing lactose, arginine and ampicillin and the tRNA producing clones able to suppress amber codons were selected by their Lac$^+$ phenotype. Positive colonies (3000 among 10^8 transformants) were selected in a second step for their ability to form blue colonies on glycerol minimal-medium containing 1 mM arginine, 1 mM IPTG, 0.5 mM x-gal (5-bromo-4-chloro-3 indolyl-ß-D-galactopyranose) and ampicillin (50 mg/mL). ß-galactosidase activity from these colonies (60 in total) was measured and the sequence of the plasmid born tRNA gene verified by the dideoxy method (9).

Purification of DHFR and identity of the yeast amber suppressor tRNA

DHFR expression was performed using a variation of the method described in (1). pDa5YC born *fol*A amber gene (the structural gene for DHFR) is expressed from the inducible p*tac* promoter.

The presence of this plasmid in *E. coli* cells is followed by chloramphenicol resistance, whereas the presence of plasmid born tRNA amber suppressor is followed by ampicillin resistance, allowing the coexistence in a single cell of two independent plasmids having identical origins of replication. Depending on the suppression efficiency of the tRNA amber suppressor measured by the ß-galactosidase assay, cells were grown in 1 to 20 L of minimal medium. The purification of DHFR was principally done using a methotrexate affinity column as described in (12, 13). The fractions with DHFR-activity were concentrated by ultrafiltration and stored at 4 °C. The protein was derivatized and sequenced by automated Edman's degradation using an Applied Biosystems 470 A Protein Sequencer equipped with a PTH 120 Analyser, allowing the identification and precise quantitative analysis of the amino acid released.

RESULTS

Construction and expression of pVBt5 amber suppressor in *E. coli*

The tRNA gene was placed under the control of the inducible *ptac* promoter located 40 bp upstream of the tRNA 5' end and the 3' terminal CCA sequence was included in the gene sequence, as it is the case for the *E. coli* tRNA genes although processing of the precursor tRNA form adds this sequence post-transcriptionally in *E. coli* cells. The CCA sequence is separated by about 170 bp from the *rrnb* operon transcription termination site. The plasmid born yeast isoleucine tRNA amber suppressor was named pVBt5 (Fig. 2). Northern blot analysis using an oligonucleotide complementary to the entire tRNA gene sequence showed that pVBt5 transformants produce a transcript of approximatively the same size as the mature yeast isoleucine tRNA (not shown). This RNA is not present in *E. coli* cells which were transformed by the pMEX vector alone and a quantitative analysis of the labeled spots indicates that the amount of expressed heterologous tRNA is about 0.25% of the total *E. coli* tRNA, even after induction by IPTG.

Suppression efficiency of pVBt5 and genetic selection of pVBt5 derived amber suppressor mutants

Plasmid pVBt5 possesses a suppression efficiency that is too low to allow growth of the *E. coli* 121 strain in the presence of lactose as the sole carbon source. The phenotype of pVBt5 transformed *E. coli* 121 cells was ligth blue colonies after several days of growth in minimal medium containing IPTG and the indicator x-gal. To select for tRNA suppressors with high efficiency, we submitted plasmid pVBt5 to random mutagenesis by passage through *mut*D5 strain. This strain is known to increase the mutation rate by a 400-1000 fold compared to a normal strain. Phagemid was prepared from the mutated plasmid and used to transfect *E. coli* 121 to obtain a high yield of transfection (100 %). We obtained about 3000 Lac^+ colonies which were blue on glycerol minimal medium supplemented with IPTG and x-gal. To eliminate the presence of revertants of the amber locus of the *lacI* portion, the recipient was retransfected by phagemid prepared from 60 Lac^+ colonies. Among these, 50 yielded blue colonies on medium containing the indicator x-gal. The level of suppression of 18 of these mutants together with pVBt5 suppressor was measured using the ß-galactosidase activity test. While the mutant forms show a suppression level near 60 %, that of pVBt5 was found below 0.1 %. The low level of heterologous pVBt5 amber suppressor is not responsible for the low level of ß-galactosidase activity since 50 % suppression can be obtained for a mutant form (i.e. pVBt5-1, see table I) while its cellular level remained unchanged as determined by Northern Blotting.

Table I. Characterization of pVBt5 mutants selected *in vivo*

	tRNA suppressor	Frequency of the in vivo mutations (%)	ß-gal. activity (%)	Amino acid acceptance
pVBt5	wild type		0,1	30 % Lys 70 % Gln
pVBt5-1	U30 ---> C30	94,5	50	Lys
pVBt5-2	G40 ---> A40 U59 ---> A59	5,5	50	Lys
pVBt5-3	G40 ---> A40	synthetic	50	Lys

ß-galactosidase activity expressed as a percentage of wild-type levels was determined for each suppressor in a strain carrying an amber mutation UAG 181 in the *I* portion of a *lacI-Z* fusion.
The amino acid acceptance of each tRNA mutant is also indicated. To measure the impact of the single G40-->A40 mutation in pVBt5-2, we synthesized a tRNA mutant bearing only G40-->A40.

Identification of the mutation responsible for the increased suppression efficiency of pVBt5 mutants

The DNA from the 18 mutants was isolated and subjected to DNA sequencing. In 17 cases a single mutation converts U30 into C30 and only one case corresponded to a double mutant G40-->A40 and U59-->A59 (pVBt5-2). In both types of mutants the original U30-G40 pair is converted to a standard Watson-Crick pair, either C30-G40 (pVBt5-1) or U30-A40 (pVBt5-3) (Table I).

In vivo identity of pVBt5 amber suppressor and derived mutant forms

To determine which *E. coli* aminoacyl-tRNA synthetase recognizes and charges the yeast isoleucine tRNA amber suppressor, we used plasmid pDa5YC coexpressed with pVBt5 by selecting for a dual chloramphenicol-ampicillin resistance. The identity of pVBt5 was Lys (70 %) and Gln (30 %) as deduced from the N-terminal sequencing data of the suppressed DHFR protein (Fig. 1). The presence of Asn at position 11 indicated that the sequenced DHFR was plasmid encoded and not the

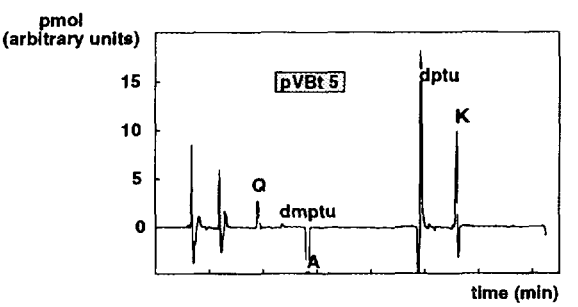

Figure 1. N-terminal sequencing data of pVBt5 suppressed DHFR.
The chromatogram visualizes the amino acid released at step 10 by Edman's degradation. It is obtained by substracting results from step 9 from those of step 10. In this way, only the amino acid(s) released at step 10 will appear on the positive scale of the ordinate, whereas the amino acid released in step 9 (Ala) is on the negative scale of the ordinate. The amino acids are written in the single letter code. Dptu (diphenylthiourea) and dmptu (dimethylphenylthiourea) are by-products of the reaction. The peak surfaces calculated directly from the integrator are in arbitrary units. They correspond in reality to 30 pmol of Gln and 70 pmol of Lys for pVBt5 amber suppressor. Sequencing yield of total amount of protein was about 20 %.

chromosomally expressed wild type DHFR. Mutant pVBt5-1 suppressor, one of the *in vivo* selected tRNA suppressor having 50 % suppression efficiency in the ß-galactosidase test is charged exclusively with Lys (Table I). Mutant pVBt5-2 having two mutations (G40-->A40 and U59-->A59), is also charged with Lys only and we could exclude that U59-->A59 mutation in the T-loop contributes to the lysine acceptance by synthesizing a tRNA suppressor mutant bearing only the G40-->A40 substitution (pVBt5-3). This mutant was as efficient as pVBt5-2 in the suppression assay and was only charged with lysine (Table I).

Modulation of the suppression efficiency and tRNA identity of pVBt5 derived mutants by a G-U pair in the anticodon stem

The above results indicate that the replacement of a U30-G40 pair in the anticodon stem of pVBt5 either by a G-C or A-U Watson-Crick pair can modulate its efficiency of suppression and to some extent its amino acid acceptance. To measure the impact of an U30-G40 pair on the suppression efficiency and glutamine acceptance of a tRNA, we have transplanted this element at identical positions in the anticodon stem of *E. coli* $tRNA^{Lys}$ and we also tested the impact of an U30-G40 inversion in pVBt5 to yield G30-U40. This inversion created three contiguous G bases at positions 29, 30 and 31 and is supposed to modify the conformation anticodon stem and/or loop as compared to the original pVBt5 conformation. The results show that the presence of a U30-G40 pair in $tRNA^{Lys}$ (pVBt5-4) does not influence either its Lys identity or its suppression efficiency (Fig. 2), whereas the inversion of the U30-G40 pair in pVBt5 prevents the mutant tRNA (pVBt5-5) to be aminoacylated by the *E. coli* GlnRS and allows charging by the *E. coli* LysRS with at least a 10 fold better efficiency as compared to pVBt5 (Fig. 2).

To better understand the structural requirements for the glutamine identity in pVBt5, we constructed a series of hybrid tRNA molecules between *E. coli* $tRNA^{Lys}$ and pVBt5 where the anticodon and acceptor stem sequences of $tRNA^{Lys}$ are progressively replaced by that of pVBt5 (Fig. 2). *E. coli* $tRNA^{Lys}$ and yeast $tRNA^{Ile}$ have D-, T- and variable loops similar in size, so that the impact of these structures on the overall tertiary structure is minimized in the chimerical molecules. In these constructions we also tested the effect of an inversion of the U30-G40 pair. In pVBt5-6 and pVBt5-7 composed of the anticodon stem sequences of pVBt5 and the rest of the tRNA molecule by that of the *E. coli* $tRNA^{Lys}$, the presence of a U30-G40 or G30-U40 pair does not affect the lysine identity of the hybrid tRNA (Fig. 2). A Gln identity is only seen when the U30-G40 pair is associated with the acceptor stems sequences of pVBt5 (pVBt5-8, Fig. 3). In pVBt5-9, where the U30-G40 pair is inverted, the Gln identity is again lost (Fig. 2).

DISCUSSION

Based on the ß-galactosidase activity tests, the aminoacylation activity of pVBt5 is 700 fold lower than that of pVBt5-1. However, care in the interpretation of the suppression results has to be taken. Indeed, only a factor of 30 separates the efficiency of pVBt5 amber suppressor from that of pVBt5-1, when comparing the levels of suppressed DHFR protein. In the *fol* gene the nucleotide context around the amber codon has been changed for maximization of suppression (1). As pVBt5 is expressed from the same plasmid, the level of tRNA used to suppress two different amber contexts

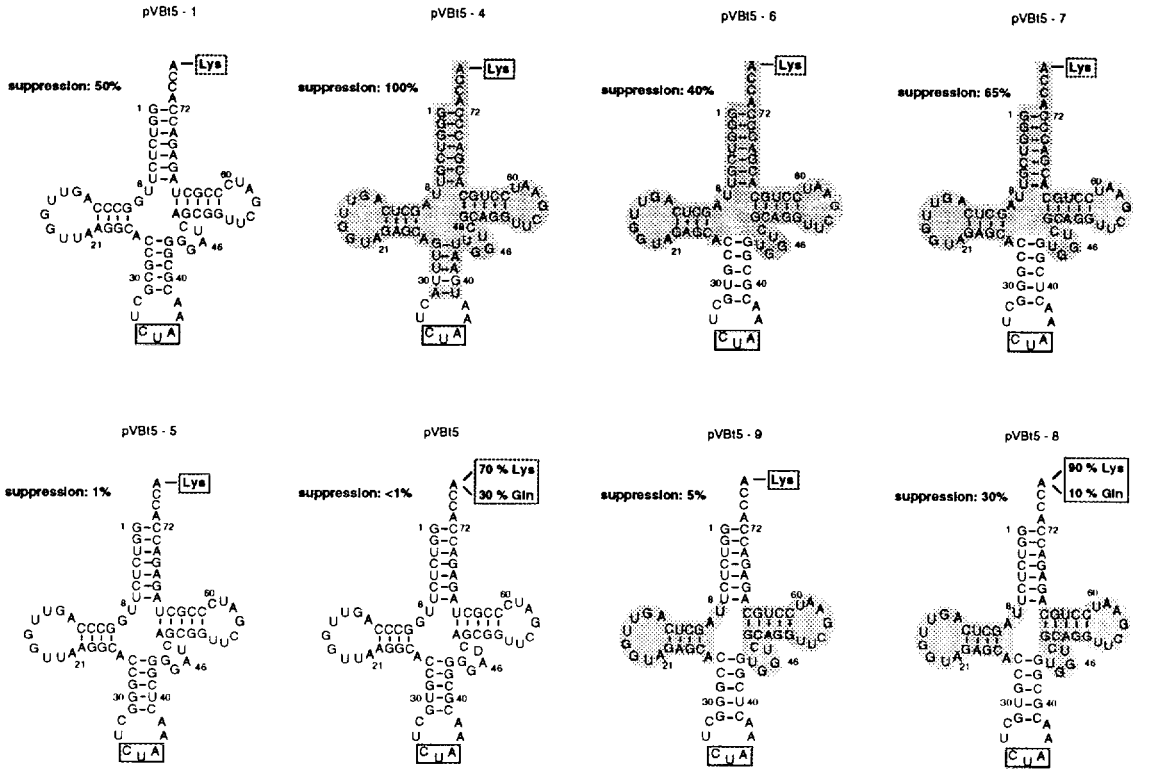

Figure 2. Summary of cloverleaf arrangements of nucleotide sequences derived from pVBt5 and *E. coli* $tRNA^{Lys}$ and identity of each construction.
The yeast sequences are indicated in bold letters and *E. coli* sequences are highlighted. Values of the efficiency of suppression are indicated for each tRNA. The amino acid attached to the CCA end has been boxed. In the cases where the tRNA has a dual amino acid acceptance, values for each amino acid are indicated. The anticodon sequence has been boxed to indicate that each tRNA represents an artificial amber suppressor tRNA.

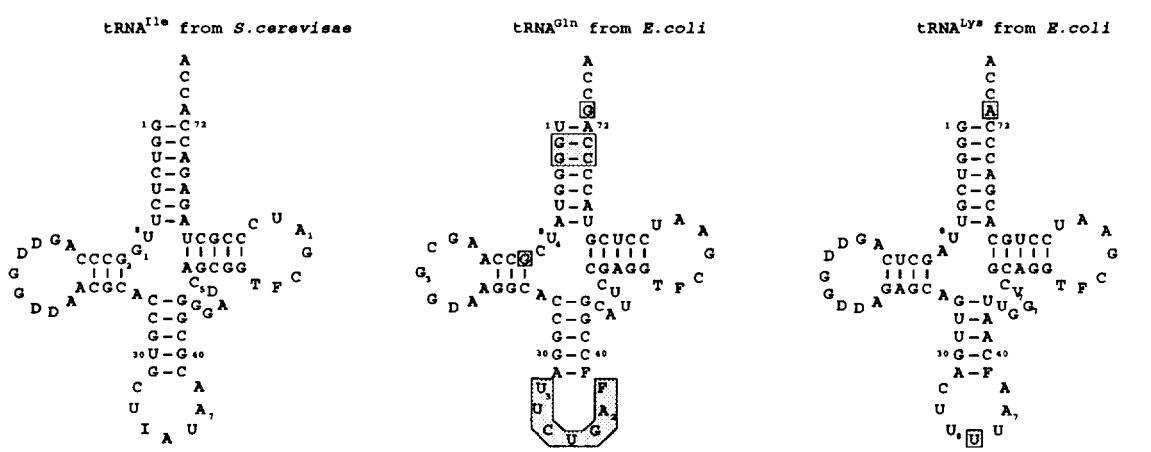

Figure 3. Cloverleaf structures of yeast $tRNA^{Ile}$, *E. coli* $tRNA^{Gln}$ and $tRNA^{Ile}$.
The figure summarizes the present knowledge on the location of identity nucleotides of *E. coli* $tRNA^{Gln}$ and $tRNA^{Ile}$ (for references see the text).

(lacZ and *fol* amber genes) is constant implying that the differences seen in the two suppression systems result from a difference in the translation efficiency and not from the plateau of aminoacylation. Assuming that pVBt5-1 is charged at a level of 100 %, we estimate the plateau of aminoacylation of pVBt5 to be 3% as deduced from the relative yields of the purified DHFR protein.

In vivo pVBt5 is charged by lysine (70%) and glutamine (30 %) in the *E. coli* cells. As far as lysine determinants are concerned, pVBt5 suppressor shares U35 and A73 lysine identity nucleotides (14) and has G1-C72, G2-C71 base pairs in common with $tRNA^{Lys}$ which constitute potential identity elements (Fig. 3). It also possesses many $tRNA^{Gln}$ identity determinants for GlnRS i.e. the G2-C71 pair in the acceptor stem and nucleotides G10, U33, C34, U35, A37 located respectively in the D-stem and anticodon loop (15, Fig. 3), but lacks the discriminatory base G73 and more important a A1-U72 pair whose melting is achieved at a lower energetic cost compared to a G1-C72 pair. This melting is necessary in order that the CCA end can adopt an hairpin structure required for an optimal fit of the terminal adenosine in the active site (16). The presence of a G1-C72 pair in pVBt5 constitutes a block for an efficient aminoacylation by GlnRS. The acceptor stem of pVBt5 has probably the major determinants for LysRS interaction, but the presence of negative determinants for LysRS located elsewhere in the molecule explains why neither LysRS nor GlnRS achieves an efficiency aminoacylation and suppression of pVBt5.

A single mutation in the anticodon stem of pVBt5 changing U30-G40 either into U30-A40 or C30-G40, converts the yeast isoleucine amber suppressor to an efficient tRNA suppressor (50%), implying a substantial increase in the efficiency of aminoacylation. The loss of the U30-G40 wobble pair is accompagnied by a loss of the Gln identity. G-U pairs are often found in tRNAs and they are known to introduce helical irregularities which can affect the interaction with proteins. As no direct contacts between the protein and nucleotides 30-40 are seen in the crystal structure of the $tRNA^{Gln}$-GlnRS complex (2, 16), this suggests that the presence of a U30-G40 pair in the anticodon stem of pVBt5 contributes indirectly to the Gln identity. To check whether a U30-G40 pair expresses a glutamine identity in the context of another anticodon stem, we replaced G30-C40 pair of *E. coli* $tRNA^{Lys}$ by U30-G40. This change modifies neither the lysine acceptance nor the suppression efficiency of a synthetic $tRNA^{Lys}$ amber suppressor (pVBt5-4) indicating that a U30-G40 pair is a weak determinant for Gln identity that necessitates the presence of other positive determinants and/or the absence of negative determinants for GlnRS in the rest of the molecule, most probably in the acceptor stem. Inverting the U30-G40 pair of pVBt5 results in a tRNA suppressor that has three consecutive G-C pairs in the anticodon stem. This tRNA (pVBt5-5) is exclusively charged with lysine and has a suppression efficiency increased by a factor of 14-fold as compared to pVBt5. The base-pair inversion has probably generated a change in the anticodon loop conformation which affects both the translational step and the aminoacylation specificity. To further confirm this observation, we made chimaera between *E. coli* $tRNA^{Lys}$ and pVBt5 where the anticodon and acceptor stems of $tRNA^{Lys}$ are progressively replaced by that of pVBt5. Here again a U30-G40 pair in the anticodon stem determines a weak $tRNA^{Gln}$ identity in the presence of the yeast isoleucine acceptor stem nucleotides, and this identity is lost when the U30-G40 pair is inverted. It is plausible that a U30-G40 pair imparts a conformation to the anticodon loop that allows a better interaction of *E. coli* GlnRS with U35 base which is the major $tRNA^{Gln}$ identity nucleotide in the anticodon region. In the

light of this result the dual amino acid identity of pVBt5 can be explained in terms of negative interactions generated by a U30-G40 pair for LysRS recognition. As our results are obtained from *in vivo* experiments, we cannot distinguish whether the U30-G40 pair influences the identity of pVBt5 by decreasing the competition with LysRS or increasing the catalytic efficiency of the pVBt5-GlnRS complex. The hypothesis of a change in the anticodon loop conformation resulting from a change in the anticodon stem is reinforced by observations from the literature. In *E. coli* initiator $tRNA^{Met}$, mutation of the three conserved G-C base pairs of the anticodon stem affects initiation of protein synthesis and conformation of the anticodon loop as shown by S1 mapping (17).

Our results showing that the activity of an anticodon is dependent on the sequence of the anticodon helix are in line with the hypothesis of an extended anticodon loop postulated by Yarus et al. (18). A particular feature associated with the presence of a G-U pair in the anticodon stem has also been noted for a mitochondrial frameshift suppressor. It was shown that a single base change from C to U at position 42 of the mitochondrial serine-tRNA (UCN) to yield a G26-U42 pair in the anticodon stem can cause frameshift suppression. This mutant tRNA also lacks the pseudouridine modification at position 27, so that an ambiguity remains whether the base change and/or the lack of modification of U27 leads to an alteration of the anticodon loop that enables this tRNA to read a four base codon UCCA (19). Whether a difference in the base modification pattern of pVBt5 derived constructions is associated with the inversion of a U-G pair in the anticodon stem remains to be determined.

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