Identity Switches between tRNAs Aminoacylated by Class I Glutaminyl- and Class II Aspartyl-tRNA Synthetases†

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ABSTRACT: High-resolution X-ray structures for the tRNA/aminoacyl-tRNA synthetase complexes between *Escherichia coli* $tRNA^{Gln}$/GlnRS and yeast $tRNA^{Asp}$/AspRS have been determined. Positive identity nucleotides that direct aminoacylation specificity have been defined in both cases; *E. coli* $tRNA^{Gln}$ identity is governed by 10 elements scattered in the tRNA structure, while specific aminoacylation of yeast $tRNA^{Asp}$ is dependent on 5 positions. Both identity sets are partially overlapping and share 3 nucleotides. Interestingly, the two enzymes belong to two different classes described for aminoacyl-tRNA synthetases. The class I glutaminyl-tRNA synthetase and the class II aspartyl-tRNA synthetase recognize their cognate tRNA from opposite sides. Mutants derived from glutamine and aspartate tRNAs have been created by progressively introducing identity elements from one tRNA into the other one. Glutaminylation and aspartylation assays of the transplanted tRNAs show that identity nucleotides from a tRNA originally aminoacylated by a synthetase from one class are still recognized if they are presented to the enzyme in a structural framework corresponding to a tRNA aminoacylated by a synthetase belonging to the other class. The simple transplantation of the glutamine identity set into $tRNA^{Asp}$ is sufficient to obtain glutaminylatable tRNA, but additional subtle features seem to be important for the complete conversion of $tRNA^{Gln}$ in an aspartylatable substrate. This study defines C38 in yeast $tRNA^{Asp}$ as a new identity nucleotide for aspartylation. We show also in this paper that, during the complex formation, aminoacyl-tRNA synthetases are at least partially responsible for conformational changes which involve structural constraints in tRNA molecules.

Specific aminoacylation of tRNAs[1] by their cognate aminoacyl-tRNA synthetases is a key step in the faithful expression of the genetic code. Correct recognition between aminoacyl-tRNA synthetase and tRNA is effected by positive and negative molecular signals specific to the given pair [reviewed in Giegé *et al.* (1993) and McClain (1993)]. Positive identity elements correspond to a limited number of nucleotides or to posttranscriptional modifications [e.g., Normanly and Abelson (1989), Schimmel (1989), Pütz *et al.* (1991), Schulman (1991), Söll (1991), and Sylvers *et al.* (1993)]. Identity elements can interact directly with specific amino acids of the synthetase. However, they can act indirectly, for example, to establish subtle structural features that allow optimal presentation of other identity elements. Negative signals or antideterminants prevent productive interactions of a tRNA with noncognate enzymes (Muramatsu *et al.*, 1988; Perret *et al.*, 1990a).

In contrast to tRNAs that all share the same global L-shaped structure, aminoacyl-tRNA synthetases are divided into two structural classes on the basis of different catalytic site organization (Cusack *et al.*, 1990; Eriani *et al.*, 1990). This structural partition is correlated with functional characteristics of these enzymes. Class I synthetases possess a Rossman fold-like catalytic site and aminoacylate their cognate tRNAs on the 2′-hydroxyl of the terminal ribose, whereas class II enzymes contain three well-conserved motifs and catalyze the reaction on the 3′-hydroxyl of the terminal sugar. In the case of *Escherichia coli* glutamine and yeast aspartate systems, the class difference is expressed by different interaction modes of the tRNA with the synthetases (Rould *et al.*, 1989; Ruff *et al.*, 1991). In both $tRNA^{Gln}$/GlnRS and $tRNA^{Asp}$/AspRS complexes the interactions are extensive over the surfaces of the two interacting macromolecules, but the recognition processes involve opposite sides of L-shaped tRNA structures (Figure 1). Whereas GlnRS interacts with nucleotides localized on the D-loop side of $tRNA^{Gln}$ and thus contacts the minor groove of the acceptor stem, AspRS recognizes nucleotides in the major groove by the variable region side of the $tRNA^{Asp}$ molecule. The conformational changes in tRNA during complex formation are also very different. Superposition of complexed $tRNA^{Gln}$ with free tRNA shows that in $tRNA^{Gln}$ the two extremities of the L-shaped structure are modified (Rould *et al.*, 1989). The first base pair of the acceptor stem is broken, and the 3′-CCA end makes a hairpin structure to adapt to the catalytic site of the enzyme (Rould *et al.*, 1989). In the case of the $tRNA^{Asp}$/AspRS complex this is not the case, but the global orientation between acceptor and anticodon branches is more constrained when comparing the complexed structure of $tRNA^{Asp}$ with the free form of tRNA (Ruff *et al.*, 1991). Finally, the dramatic conformational changes in the anticodon loops facilitate the interactions with the synthetase counterparts (Rould *et al.*, 1991; Cavarelli *et al.*, 1993).

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[1] Abbreviations: tRNA, transfer RNA; aaRS, aminoacyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; AspRS, aspartyl-tRNA synthetase.

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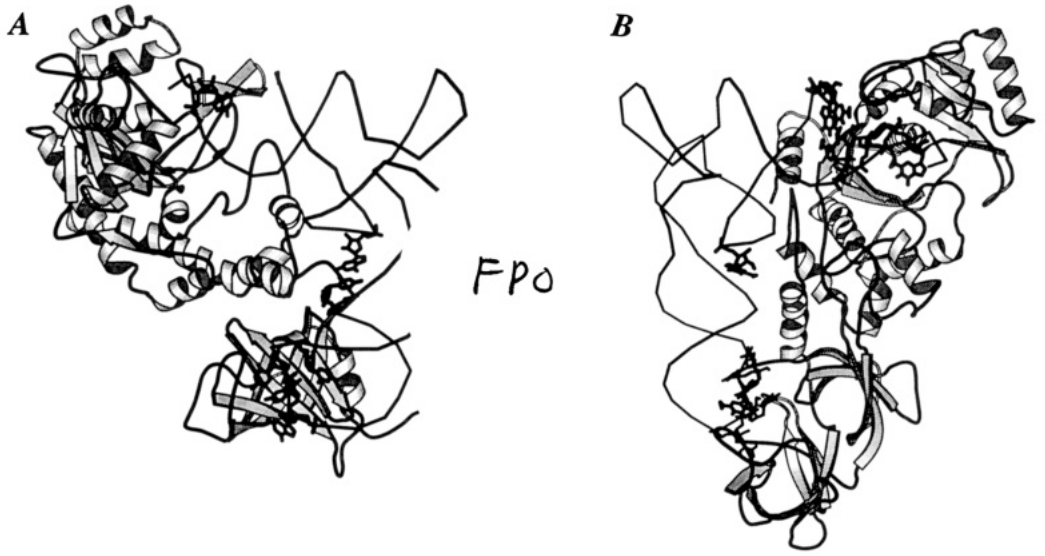

FIGURE 1: Crystal structure of yeast tRNA^{Asp}/AspRS (A) and *E. coli* tRNA^{Gln}/GlnRS (B) complexes. This figure emphasizes the opposite sides of recognition of tRNAs by the enzymes. For simplicity, only one monomer of AspRS interacting with one tRNA is represented. The figure displays also the identitiy nucleotides in both tRNAs, including the novel aspartate determinant at C38 discovered in the course of this study. The drawings (courtesy of Jean Cavarelli, Strasbourg) were computed using the coordinates from Cavarelli and Moras (1993) and the program Molscript (Kraulis, 1991).

The concept of identity implies that transplantation of identity sets from one tRNA to another one confers the new specificity to the host tRNA, a fact verified explicitly *in vitro* in many cases [reviewed in Giegé *et al.* (1993)]. Since optimal catalytic efficiency of tRNA aminoacylation requires optimal presentation of identity elements to the amino acids present on the synthetase, the question arises whether peculiar sequence combinations in tRNA have been retained by evolution, which would prevent cross-interactions between tRNAs and synthetases from both classes. One straightforward way to answer this question is to study the efficiency of identity set transplantation within a tRNA specific for an enzyme of class I into a tRNA framework of a tRNA specific for a synthetase of class II and *vice versa*. In this paper we investigate the potentiality of cross-transplantation and cross-aminoacylation between the *E. coli* class I glutamine and yeast aspartate class II systems for which, in addition to X-ray structures, identity sets are known (Jahn *et al.*, 1991; Hayase *et al.*, 1992; Pütz *et al.*, 1991).

In the aspartate and glutamine systems, identity elements are scattered within the same discrete regions of the tRNAs, namely, the acceptor stem, the D-loop, and the anticodon loop (Figures 1 and 2A). In the yeast aspartate system tRNA identity is governed by G73 in the acceptor stem, the base pair G10·U25 in the D-stem, and the three anticodon bases (G34, U35, and C36) (Pütz *et al.*, 1991) (Figure 2A1). In *E. coli* tRNA^{Gln}, identity nucleotides correspond to the discriminator base G73, the three first base pairs in the acceptor stem (U1·A72, G2·C71, and G3·C70), nucleotide G10 in the D-stem, and nucleotides 34–38 in the anticodon loop (C34, U35, G36, A37, and U38) (Jahn *et al.*, 1991; Hayase *et al.*, 1992) (Figure 2A2). Thus, both tRNAs share three identity nucleotides which are the discriminator base G73, nucleotide G10, and the central position of the anticodon U35. In both tRNAs, most of the identity nucleotides interact directly with amino acids in the synthetase. Only the G10·U25 pair in tRNA^{Asp} is involved in the tertiary structure and plays an indirect role (Ruff *et al.*, 1991; Rudinger *et al.*, 1992). The experiments which will be presented here have been designed in such a way that reciprocal conversion of identities would be progressive; i.e., a progressive acquisition of glutamine identity by tRNA^{Asp} with a concomitant loss of its aspartate identity and *vice versa*.

Our results demonstrate that efficient transplantations from class I toward class II systems and *vice versa* are possible. Furthermore, they show, as anticipated, that the acquisition of the new specificities can be progressive. However, conversion of a glutamine into an aspartate acceptor requires less sequence changes within the tRNA than the converse transplantation, aspartate to glutamine. These observations and their implication for a better understanding of tRNA identities will be discussed. These studies also revealed a new aspartate identity element.

MATERIALS AND METHODS

Materials. Oligodeoxynucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer using the phosphoramidite method and purified by HPLC on a Nucleosil 120-5-C18 column (Bischoff Chromatography, Zymark-France, Paris). L-[^{3}H]Aspartic acid (43 Ci/mmol) and L-[^{3}H]-glutamine (53 Ci/mmol) were from Amersham France (Les Ulis), and yeast AspRS (Lorber *et al.*, 1983) and T7 RNA polymerase (Wyatt *et al.*, 1991) were purified as described previously. Pure *E. coli* GlnRS and the plasmid encoding wild-type tRNA^{Gln} were prepared as described (Perona *et al.*, 1989; Jahn *et al.*, 1991). Restriction enzymes *Bst*N1, *Hin*dIII, and *Bam*H1 and T4 polynucleotide kinase were from New England Biolabs (Beverly, MA). T4 DNA ligase was from Boehringer Mannheim (Meylan, France).

Cloning and in Vitro Transcription. All tRNAs used in this work have been obtained by *in vitro* transcription of synthetic genes. Each of these genes corresponds to the T7 RNA polymerase promoter region directly connected to the downstream tRNA sequence. The tRNA genes were constructed and cloned according to established methods (Sampson & Uhlenbeck, 1988; Perret *et al.*, 1990b). *In vitro* transcriptions were performed in reaction mixtures containing 40 mM Tris-HCl, pH 8.1 (at 37 °C), 22 mM $MgCl_2$, 5 mM dithioerythritol, 0.01% Triton X-100, 1 mM spermidine, 4 mM each nucleoside triphosphate, 5 mM GMP, 0.1 μg/μL linearized plasmid, and an adequate amount of T7 RNA polymerase. Incubations were for 3 h at 37 °C, and reactions

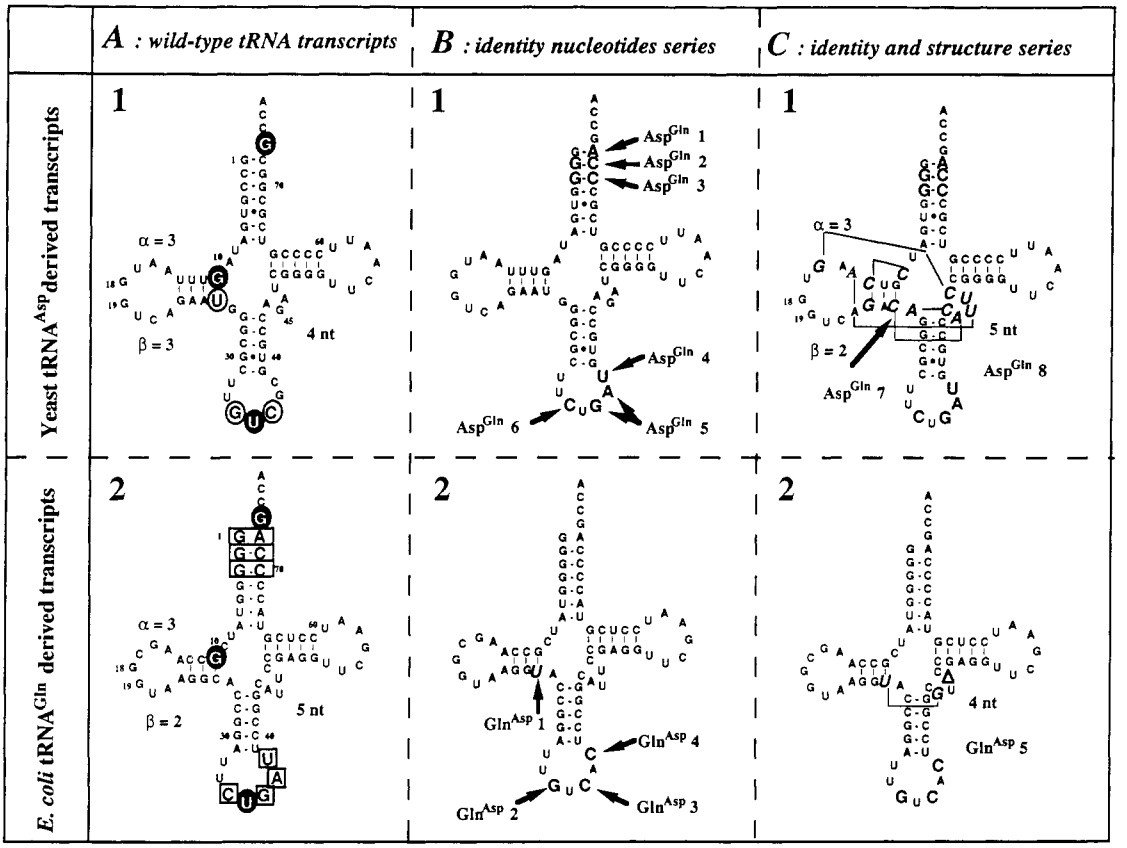

FIGURE 2: Cloverleaf structures of yeast $tRNA^{Asp}$ and *E. coli* $tRNA^{Gln}$ (A), and of a series of variants (B, C) derived from both tRNAs. Panel A represents the wild-type sequences of $tRNA^{Asp}$ (A1) and $tRNA^{Gln}$ (A2) transcripts with identity nucleotides highlighted and structural features in D-loops indicated. Aspartate identity positions (Pütz *et al.*, 1991) are circled, and glutamine identity positions (Jahn *et al.*, 1991) are boxed. Note that three elements are common to both sequences. For transcriptional reasons, both transcripts have base pairs 1·2 changed as compared to the natural tRNAs: $tRNA^{Asp}$ transcript has a G·C sequence instead of U·A and $tRNA^{Gln}$ transcript a G·A pair instead of a U·A pair. These changes do not influence the aminoacylation properties of these transcripts. Numbering of residues and sequence data are from the compilation by Steinberg *et al.* (1993). Panel B displays sequence variants derived from $tRNA^{Asp}$ (B1) and $tRNA^{Gln}$ (B2). Progressive integration of identity elements from the donor into the host tRNA is indicated with large characters; arrows correspond to the names of the variants (for explanation see the text and Figure 3). Panel C represents cloverleaf structures of $tRNA^{Asp}$ (C1) and $tRNA^{Gln}$ (C2) derived variants presenting alterations at both the sequence and the structural levels. Variants $Asp^{Gln}{}_7$ and $Asp^{Gln}{}_8$ in panel C1 contain respectively the (G10·C25)A45 triple as in $tRNA^{Gln}$ and all the glutamine identity nucleotides as well as the nucleotides involved in the network of tertiary interactions found in $tRNA^{Gln}$; variant $Gln^{Asp}{}_5$ in panel C2 is a variable region variant of $tRNA^{Gln}$ containing the complete aspartate identity set including the $tRNA^{Asp}$ specific triple (G10·U25)G45. In both panels B and C, identity nucleotides are in boldface and structural elements in italic.

were stopped by phenol/chloroform extraction. Full-length transcripts correctly ending with the CCA sequence were purified by preparative electrophoresis on 12% polyacrylamide denaturing gels followed by electroelution (Schleicher and Schuell, Dassel, Germany). Concentration of stock solutions of tRNA transcripts was determined by absorbance measurements at 260 nm.

Aminoacylation Reactions. All aminoacylation reactions were performed in the assay buffer containing 30 mM HEPES–KOH, pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, 25 mM KCl, 5 mM dithiothreitol, 100 μM [^{3}H]-labeled amino acids, and appropriate amounts of tRNA transcript and synthetase. Transcripts were renatured before aminoacylation by heating to 65 °C for 90 s and slow cooling to room temperature. AspRS was diluted in bovine serum albumin at 5 mg/mL whereas GlnRS was diluted extemporaneously in water. Incubations were at 37 °C, and aminoacylated tRNA samples were quenched and counted as described (Perret *et al.*, 1990b). The kinetic constants were derived from Lineweaver–Burk plots. They represent an average of at least two independent experiments. Variants, for which k_{cat}/K_M were not determinable, gave plateau levels in the presence of 200 nM AspRS or 250 nM GlnRS in the assay conditions above.

Interpretation of Kinetic Data. The efficiency of aminoacylation of variants respective to the wild-type transcripts was appreciated by comparison of the Michaelis–Menten kinetic parameters and in particular by comparison of the specificity constants k_{cat}/K_M. Thus, $(k_{cat}/K_M)_{relative} = (k_{cat}/K_M)_{variant}/(k_{cat}/K_M)_{wild\text{-}type}$. A more intuitive number, $\mathcal{L}$, indicates the loss in aminoacylation efficiency of a variant as compared to wild-type. It corresponds to the inverse of $(k_{cat}/K_M)_{relative}$ (Pütz *et al.*, 1993). Conversely, $\mathcal{G}$ corresponds to the gain in efficiency and is calculated by the ratio between $\mathcal{L}_{variant(a)}/\mathcal{L}_{variant(b)}$, variant (a) being less well aminoacylated than variant (b).

RESULTS

Design of $tRNA^{Gln}$ and $tRNA^{Asp}$ Variants. Thirteen tRNA variants derived from *E. coli* $tRNA^{Gln}$ or yeast $tRNA^{Asp}$ have been designed in which identity sets and structural features from one species have been inserted progressively into the other (Figure 2). They include ten sequence mutants[2] called $Asp^{Gln}{}_1$ to $Asp^{Gln}{}_6$ or $Gln^{Asp}{}_1$ to $Gln^{Asp}{}_4$ (Figure 2B) and three structural mutants called $Asp^{Gln}{}_7$, $Asp^{Gln}{}_8$, and $Gln^{Asp}{}_5$ (Figure

[2] Names of variants: e.g., in $Asp^{Gln}{}_3$, Asp is the basic sequence and structure of the host tRNA; Gln indicates the origin of the integrated identity nucleotides and/or structural elements; and 3 indicates the third variant of a series.

Table 1: Kinetic Parameters for Aminoacylation of Transcripts Derived from Yeast $tRNA^{Asp}$ and *E. coli* $tRNA^{Gln}$

transcripts	aspartylation K_M (nM)	aspartylation k_{cat} (s^{-1})	aspartylation k_{cat}/K_M (10^{-3}-fold)	aspartylation $\mathcal{L}$	glutaminylation K_M (nM)	glutaminylation k_{cat} (s^{-1})	glutaminylation k_{cat}/K_M (10^{-3}-fold)	glutaminylation $\mathcal{L}$
Identity Nucleotides Series								
Asp w.t.	36	1.22	33.9	1	nm[a]	nm	nm	$>10^6$
Asp^{Gln_1}	100	1	10	3.5	nm	nm	nm	$>10^6$
Asp^{Gln_2}	80	1.1	13.7	2.4	nm	nm	nm	$>10^6$
Asp^{Gln_3}	100	1	10	3.5	nm	nm	nm	$>10^6$
Asp^{Gln_4}	470	0.75	1.6	21	nm	nm	nm	$>10^6$
Asp^{Gln_5}	620	0.015	0.02	1400	8000	0.1	0.012	180
Asp^{Gln_6}	1900	0.01	0.0052	6300	330	0.87	2.63	0.85
Gln w.t.	nm	nm	nm	$>10^6$	400	0.9	2.25	1
Gln^{Asp_1}	nm	nm	nm	$>10^6$	600	0.47	0.78	2.9
Gln^{Asp_2}	nm	nm	nm	$>10^6$	3000	0.19	0.63	35
Gln^{Asp_3}	5500	0.01	0.0018	18645	nm	nm	nm	$>10^6$
Gln^{Asp_4}	1640	1.75	1.05	32	22500	0.0013	0.000058	38900
Identity and Structure Series								
Asp^{Gln_7}	nm	nm	nm	$>10^6$	500	1	2	1.12
Asp^{Gln_8}	nm	nm	nm	$>10^6$	1900	0.17	0.009	25
Gln^{Asp_5}	1300	1.4	1.07	32	70000	0.003	0.000043	52500

[a] nm, not measurable.

2C). For simplicity the names of variants are restricted to the name of the host molecule (e.g., Asp, Gln) and the integrated new specificity element from the other tRNA is written in smaller superscripted characters. As controls for optimal glutamine and aspartate charging activities, we used unmodified wild-type tRNA transcripts, derived from $tRNA^{Asp}$ and $tRNA^{Gln}$ sequences. The first base pair (U1·A72) was replaced by G1·C72 and G1·A72 pairs, respectively (Figure 2A). These changes do not affect the aminoacylation of both tRNAs but improve transcription efficiency (Perret *et al.*, 1990b; Jahn *et al.*, 1991).

In both aspartate or glutamine tRNA-derived sets of mutants, progressive integration of identity nucleotides has been performed with the aim of obtaining tRNAs with partial double identities. To achieve these transformations, positions that were first transplanted were not involved in the specificity of the host tRNA. Thus, integration of glutamine identity nucleotides into $tRNA^{Asp}$ was begun by transplantation of the $tRNA^{Gln}$ acceptor stem identity elements at positions which are not involved in the aspartate identity (Asp^{Gln_1} to Asp^{Gln_3}). In the case of anticodon identity positions which are involved in the identity of both systems, the order of mutations was chosen on the basis of their kinetic importance [k_{cat}/K_M ratios according to Jahn *et al.* (1991) and Pütz *et al.* (1991)] evaluated on variants presenting single mutations at these positions. Thus, in variants Asp^{Gln_4} to Asp^{Gln_6} and Gln^{Asp_2} to Gln^{Asp_4} the order of integration was from the weakest to the strongest identity determinants.

Glutaminylation and Remaining Aspartylation Activities of Transplanted Yeast $tRNA^{Asp}$ Variants. Yeast $tRNA^{Asp}$ transcript contains already three identity elements (G73, G10, and U35) of the ten required for an optimal glutaminylation (Figure 2A) but is not recognized by GlnRS (Table 1). The series of transcripts derived from $tRNA^{Asp}$ has been tested for its acquisition of glutaminylation specificity and its concomitant loss of aspartylation activity. The corresponding kinetic data are displayed in Table 1. Replacement of C72 by A72 (Asp^{Gln_1}) shows that the presence of a non-Watson–Crick base pair at the extremity of the $tRNA^{Asp}$ acceptor stem has a weak effect on aspartylation efficiency. The k_{cat} is unchanged while this mutation increases the K_M only 3 times as compared to the wild-type $tRNA^{Asp}$ transcript. The additional replacement of the second (C2·G71) and third (C3·G70) base pairs of $tRNA^{Asp}$ by G2·C71 and G3·C70 in Asp^{Gln_2} and Asp^{Gln_3}, respectively, does not reduce their aspartylation efficiency. However, the combined presence of this partial glutamine identity set (six elements out of ten) within the acceptor stem of $tRNA^{Asp}$ does not confer glutaminylation activity to the corresponding Asp^{Gln} transplants ($\mathcal{L} > 10^6$). We do not exclude aminoacylation activities with higher losses which were not systematically searched, because the main purpose of this work was not to detect minute residual activities but to search for the most efficient specificity changes.

When, in addition to the previous mutations introduced at the level of the acceptor stem, C38 is mutated into U38 (Asp^{Gln_4}), glutaminylation is still not detected but, unexpectedly, the aspartylation properties become affected. The 20-fold loss in aspartate specificity of this mutant is mainly due to a K_M effect which is increased 5-fold and which likely weakens the affinity of the molecule for AspRS. The concomitant integration of two further nucleotides, A37 and G36 (mutant Asp^{Gln_5}), decreases particularly the catalytic rate (k_{cat}) of aspartylation (50-fold) whereas the K_M does not vary. Glutaminylation becomes detectable in this mutant. Indeed, a dramatic improvement of the new specificity appears ($\mathcal{S} > 530$). In other words, this variant is only 180-fold less efficient than the wild-type substrate. This effect is produced by both K_M and k_{cat} effects.

Complete acquisition of glutaminylation was achieved for mutant Asp^{Gln_6} where G34 has been changed to C34. This mutant possesses the complete glutamine identity set. The K_M and catalytic constants of this mutant, similar to those of the wild-type $tRNA^{Gln}$, suggest that its aminoacylation occurs with the same mechanism. It is noteworthy that this mutant still retains a significant aspartylation activity with a loss of about 6300-fold of efficiency. This behavior is obviously related to the presence of three out of five aspartate identity elements (Pütz *et al.*, 1991).

Aspartylation and Remaining Glutaminylation Activities of Transplanted E. coli $tRNA^{Gln}$. The wild-type $tRNA^{Gln}$ shares two of the five identity elements determined for aspartylation (Pütz *et al.*, 1991). Although G73 and U35 have strong effects on the aminoacylation efficiency by yeast AspRS, they do not confer any detectable aspartylation capacity to the wild-type $tRNA^{Gln}$ transcript.

The conversion of *E. coli* $tRNA^{Gln}$ into yeast $tRNA^{Asp}$ was begun by transplantation of the G10·U25 base pair specific for aspartylation. The corresponding variant Gln^{Asp}_1 is not affected in glutaminylation (Table 1) and is not chargeable by AspRS, showing that three out of five identity elements are not sufficient to allow aspartylation of Gln^{Asp}_1. Further incorporation of G34 into host Gln^{Asp}_1 anticodon loop leads to variant Gln^{Asp}_2. Glutaminylation of this variant is decreased significantly as compared to wild-type $tRNA^{Gln}$ and to Gln^{Asp}_1. A loss of aminoacylation efficiency relative to wild-type $tRNA^{Gln}$ of 35-fold is due to both an increase of K_M by a factor 5 and a decrease of the catalytic constant of about 7.5-fold. Variant Gln^{Asp}_2 is still not recognized by AspRS.

Integration of the fifth aspartate identity nucleotide into Gln^{Asp}_2 leads to variant Gln^{Asp}_3, which is an active substrate for AspRS. Surprisingly, however, the aspartylation efficiency of this transcript is still reduced 18 600-fold in comparison to that of wild-type $tRNA^{Asp}$. This loss in efficiency is linked to a 152-fold decreased affinity and a 122-fold decreased catalytic rate. In parallel to the beneficial effect on aspartylation, the presence of G36 in Gln^{Asp}_3 leads to a complete loss of detectable glutaminylation.

In order to obtain a $tRNA^{Gln}$-derived transcript with increased aspartylation capacity, an additional base mutation at position 38 was tested. The reason for this choice is linked to the effect of position 38 on aspartylation of variant Asp^{Gln}_4. Replacement of U38 by C38 leads to an increase in aspartylation efficiency by 590-fold as compared to Gln^{Asp}_3. The K_M of variant Gln^{Asp}_4 is still 45-fold higher than in the case of wild-type $tRNA^{Asp}$, but the catalytic rate of the reaction becomes equivalent. Interestingly, Gln^{Asp}_4 again acquires glutaminylation activity, which was completely lost in the Gln^{Asp}_3 variant. This recovered catalytic activity is weak however since it is reduced 39 000-fold as compared to that of wild-type $tRNA^{Gln}$ transcript (Table 1).

Aminoacylation Properties of Transplanted tRNAs with Structural Modifications. Structural features present in native $tRNA^{Asp}$ and $tRNA^{Gln}$ differ to some extent. These variations concern the tertiary interactions in the D- and T-loop regions of the L-shaped structure of tRNAs. $tRNA^{Asp}$ possesses an $\alpha 3\beta 3$ D-loop, 4 nucleotides in the variable loop, and 4 base pairs in the D-stem, whereas $tRNA^{Gln}$ has an $\alpha 3\beta 2$ D-loop, a 5-nucleotides-long variable loop, and 3 Watson–Crick and 1 non-Watson–Crick base pairs in the D-stem [see Giegé *et al.* (1993)]. Significant structural changes, directly correlatable to the building up of the D-loop domains α and β, have been demonstrated in a previous work (Perret *et al.*, 1992). Moreover, all nucleotides involved in tertiary interactions are different in both tRNAs except G10.

The first structural change introduced into host Asp^{Gln}_6 was to replace the aspartate identity element G10·U25 by G10·C25. The corresponding variant Asp^{Gln}_7 was no longer a substrate for AspRS (Table 1), and as expected since base pair C25 is not involved in glutamine identity (Hayase *et al.*, 1992), no changes were observed at the level of glutaminylation. The kinetic parameters for glutaminylation of Asp^{Gln}_6 and Asp^{Gln}_7 are very similar.

To test the role of the glutamine-specific structural framework on the expression of glutamine identity, all the tertiary interactions derived from the $tRNA^{Gln}$ sequence (Yaniv & Folk, 1975) were introduced into the $tRNA^{Asp}$ mutant already containing the complete identity set for glutaminylation (Asp^{Gln}_8). Interestingly, glutaminylation of Asp^{Gln}_8 (Figure 2C1) is only 25-fold as efficient as variants Asp^{Gln}_6 and Asp^{Gln}_7, which were as efficient glutamine acceptors as wild-type $tRNA^{Gln}$. Both affinity and catalytic rate were reduced 4- to 5-fold (Table 1). This variant is not aspartylatable.

Along the same line, Gln^{Asp}_4 containing the complete aspartate identity set was further engineered at the structural level. Two mutations in the variable loop were introduced in order to recover the triple tertiary interaction (G10·U25)G45 typical for $tRNA^{Asp}$ (Gangloff *et al.*, 1971; Westhof *et al.*, 1985). Thus, Gln^{Asp}_5 contains G45 instead of A45 and a variable loop reduced from 5 to 4 nucleotides (deletion of U47). Kinetic parameters of this variant are equivalent to those of Asp^{Gln}_4 (Table 1).

DISCUSSION

Interchangeability of tRNAs for Class I and Class II Synthetases. The results of the present work show that switching the identity of a tRNA specific for a class I synthetase into an identity corresponding to a tRNA specific for a class II synthetase, and *vice versa*, is feasible. This conclusion was obtained with the yeast aspartate (class II) and *E. coli* glutamine (class I) systems. Interclass transplantations of identity sets conferring new identities have already been reported a number of times, but their converse transplantations have not yet been studied [e.g., Normanly *et al.* (1986), Rogers and Söll (1988), Hou *et al.* (1989), McClain *et al.* (1990), Himeno *et al.* (1990), and Achsel *et al.* (1993)]. We show here that the simple transplantation of the identity set from *E. coli* $tRNA^{Gln}$ into the yeast $tRNA^{Asp}$ framework leads to an optimal substrate for the glutaminylation reaction. The variant molecule Asp^{Gln}_6, possessing the complete glutamine identity set as determined previously (Jahn *et al.*, 1991; Hayase *et al.*, 1992), presents kinetic parameters for glutaminylation identical to those of wild-type $tRNA^{Gln}$, clearly demonstrating that the chimer contains sufficient information for optimal glutaminylation. The symmetric experiment where the identity set from yeast $tRNA^{Asp}$ (Pütz *et al.*, 1991) was transferred into *E. coli* $tRNA^{Gln}$ (Gln^{Asp}_3) did not confer complete aspartylation efficiency ($\mathcal{L} = 18\ 640$). An additional mutation at position 38 (U to C) increases 590-fold the efficiency of aminoacylation and confers to this molecule (Asp^{Gln}_4) an efficiency only 32-fold below that of the wild-type $tRNA^{Asp}$ with a similar k_{cat} but an increased K_M.

A detailed analysis of the relationships between the kinetic properties of the various mutants tested for their aspartylation and glutaminylation properties, summarized in Figure 3, reveals (i) that progressive exchanges of identities could be obtained and that mutants possessing a dual specificity are generated, (ii) that incorporation of some identity elements has discrete effects whereas others, especially changes in the anticodon, contribute much more to the change of specificity, (iii) that nucleotide 38 has a dual function since it corresponds to a heretofore unknown aspartate identity element and since it is involved in an anticooperative interaction in $tRNA^{Gln}$, (iv) that partial identity sets are expressed differently according to the host tRNA framework, and (v) that conformational features do not enhance the expression of identity sets but unexpectedly can decrease them.

Altogether, our data indicate that evolution has not selected features in tRNAs that would restrict possible cross-interactions with synthetases from the two classes or from both prokaryotic and eukaryotic kingdoms. However, as experiments were done with transcripts, possible effects of differing levels of tRNA modification would not be detected.

Progressivity in Exchanges of Identities. Figure 3 shows in a summarized representation that losses ($\mathcal{L}$ values) in one

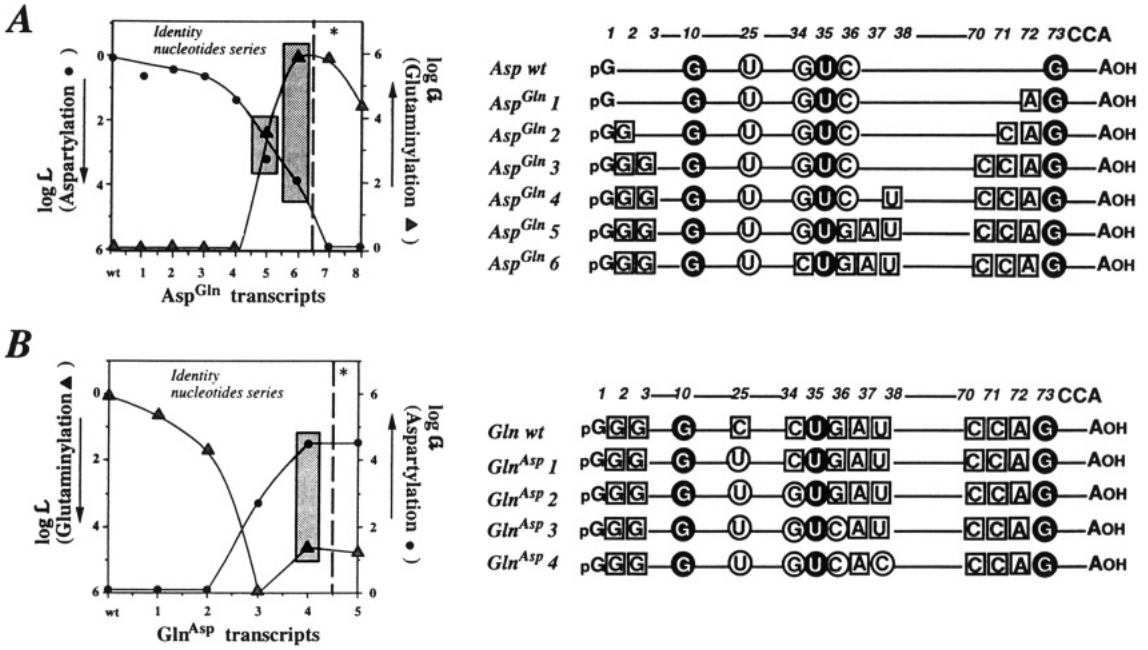

FIGURE 3: Summary of the functional data of tRNA^{Asp} (A) and tRNA^{Gln} (B) variants, emphasizing the progressive loss of the primitive specificity (expressed as log $\mathcal{L}$ values) and the correlated gain of the new specificity (expressed as log $\mathcal{G}$ values). The shaded boxes show the kinetic properties of the variants with dual aspartate and glutamine specificities. The sequence alterations, for identity nucleotide transplantations, introduced in both tRNA^{Asp} (Asp^{Gln} series) and tRNA^{Gln} (Gln^{Asp} series) are indicated at the right-hand side of the figure: aspartate determinants are light circled and glutamine determinants, differing from aspartate determiants, are boxed. The G10, U35, and G73 residues which are the determinants common to all molecules are displayed as dark circles.

aminoacylation specificity are progressive and directly correlated to progressive gains ($\mathcal{G}$ values) of the other specificity. In other words, progressive integration of glutamine identity nucleotides into tRNA^{Asp} transcripts leads to progressive acquisition of glutamine properties, and at the same time, progressive diminution of the number of aspartate identity elements leads to a progressive loss in aspartylation ability of the corresponding transcripts (Figure 3A). This progression holds true for variants derived from tRNA^{Gln} losing glutamine and acquiring aspartate specificity (Figure 3B). Progressive exchanges of specificities could be obtained by logical order of introduction of identity elements. This was achieved by taking into account the strength of individual mutations (expressed as $\mathcal{L}$ values) (Jahn *et al.*, 1991; Pütz *et al.*, 1991) and mutating first those positions which are not overlapping in the aspartate and glutamine identity sets.

In the case of the Asp^{Gln} series, the first glutamine identity elements introduced into host tRNA^{Asp} were those of the acceptor stem (A72, G2·C71, and G3·C70 in variants Asp$^{Gln}{}_1$, Asp$^{Gln}{}_2$, and Asp$^{Gln}{}_3$, respectively). Whereas these positions were shown to be relatively weak glutamine identity determinants in tRNA^{Gln} ($2 < \mathcal{L} < 1650$) (Jahn *et al.*, 1991), they are not involved in aspartate identity (Pütz *et al.*, 1991). Mutations at these positions in the tRNA^{Asp} context had, as expected, no significant effect on aspartylation (the peculiar case of variant Asp$^{Gln}{}_1$ will be discussed below). Incorporation of identity nucleotides from the anticodon loop, which were shown to correspond to strong identity determinants in both systems, leads to detectable effects, both for losses in aspartylation and for gains in glutaminylation activity. Starting from the 3′-end of the loop, incorporation of nucleotide U38 (Asp$^{Gln}{}_4$) shows a negative effect on aspartylation and has still no effect on glutaminylation, suggesting that C38 contributes to aspartate identity (see below). Additional changes at nucleotides 37, 36, and finally 34 conferred optimal glutaminylation properties to tRNA^{Asp} and a further decreased aspartylation capacity. Indeed, the kinetic parameters for glutaminylation of Asp$^{Gln}{}_6$ are the same as those obtained for wild-type tRNA^{Gln}, while the loss in aspartylation efficiency of this variant is 6300-fold as compared to wild-type tRNA^{Asp}.

Conversion of tRNA^{Gln} to an efficient aspartate acceptor was obtained following the same line (Figure 3B). Residues G73 and U35 were already present; thus, the first incorporation of aspartate identity elements corresponded to U25 (Gln$^{Asp}{}_1$), an identity element which was shown to have the lowest effect after mutation in the tRNA^{Asp} framework (Pütz *et al.*, 1991). Further addition of residue G34 (Gln$^{Asp}{}_2$) did not allow the transition state of the aspartylation reaction to be overcome (within the limits of our assays) but lowered somehow the glutaminylation ability of the tRNA. Variant Gln$^{Asp}{}_3$, presenting the complete aspartate identity set as defined by Pütz *et al.* (1991), acquired an aspartylation capacity and concomitantly lost its glutaminylation ability ($\mathcal{L} > 10^6$) although it presents almost all of the glutamine identity elements (8 out of 10). Complete aspartylation of a tRNA^{Gln}-derived transcript was attempted by incorporation of nucleotide C38 which was shown to be of some importance to aspartylation in the studies on Asp^{Gln} variants, and indeed, improved aspartylation ability was obtained for variant Gln$^{Asp}{}_4$. Interestingly, this variant loses an element important for glutamine identity within the anticodon loop and, however, recovers concomitantly some glutamine activity. Thus, this variant (Gln$^{Asp}{}_4$) is a substrate for both aminoacyl-tRNA synthetases studied here.

As highlighted in Figure 3, variants Asp$^{Gln}{}_5$, Asp$^{Gln}{}_6$, and Gln$^{Asp}{}_4$ are efficient substrates for both yeast AspRS and *E. coli* GlnRS and thus show a double specificity of aminoacylation. This is obviously due to the presence of sufficient aspartate and glutamine identity elements within these variants. Aminoacylation efficiencies with both enzymes are equivalent only in the case of Asp$^{Gln}{}_5$. In the two other variants, one of the specificities is expressed much more than the other. tRNAs bearing two or even three identities at once have already

been described a number of times [see Frugier *et al.* (1993) and references therein].

Importance of Anticodon Regions. As discussed above, the first mutants studied either in the aspartate to glutamine conversion (Asp^{Gln}_1 to Asp^{Gln}_4) or in the glutamine to aspartate conversion (Gln^{Asp}_1 and Gln^{Asp}_2) do not show drastic changes in their aminoacylation behaviors (Table 1, Figure 3). Dramatic gains or losses in aminoacylation efficiences appear only with variants where, in addition to changes performed in the acceptor or D-stems, changes in the anticodon domain have been performed. Losses in efficiencies of variants of $tRNA^{Gln}$ or $tRNA^{Asp}$ presenting individual mutations at the level of anticodon or anticodon loop nucleotides were already shown previously to be much higher than for other identity elements in either system. In the case of $tRNA^{Gln}$ they vary from 66- to 27 800-fold whereas other determinants lead to losses between 2- and 1650-fold (Jahn *et al.*, 1991; Hayase *et al.*, 1992). In the case of $tRNA^{Asp}$, single anticodon nucleotide mutations lead to losses varying between 19- and 530-fold, while mutations elsewhere led to losses not exceeding 200-fold (Pütz *et al.*, 1991). The results of the progressive transplantation experiments described here confirm the large contribution of the anticodon domain nucleotides, and especially of the anticodon triplet, to the expression of both glutamine and aspartate identities. This correlates well with the large conformational changes observed in the anticodon loops of the glutamine- and aspartate-specific tRNAs complexed to their cognate synthetases (Rould *et al.*, 1989; Ruff *et al.*, 1991), allowing optimal contacts between the identity nucleotides and the conterparts at the level of the synthetase.

The contribution of the discriminator base to the level of identity expression within either host tRNA cannot be measured here since both $tRNA^{Asp}$ and $tRNA^{Gln}$ possess in common residue G73. This nucleotide is important in both aspartate and glutamine systems. In the first one, G73 contacts AspRS (Ruff *et al.*, 1991), while in the second one, it contributes to the special positioning of the CCA toward GlnRS (Rould *et al.*, 1989). This important contribution of the discriminator base to both systems has been confirmed recently by the aminoacylation properties of minihelices mimicking the aminoacyl-acceptor arm from both tRNAs (Frugier *et al.*, 1994; Wright *et al.*, 1993). The contribution of the discriminator base to either aminoacylation specificities could be estimated by progressive transplantation of identity elements into a third host tRNA where residue 73 is different from G. It remains unclear if progressive transplantation experiments of either glutamine or aspartate identity elements into a host tRNA possessing a nonconserved discriminator base would designate the anticodon or the discriminator base as the most important identity element.

A Dual Function for Nucleotide 38. Despite the presence of the complete identity set for aspartylation, Gln^{Asp}_3 was still 18 600-fold less well aminoacylated by AspRS than the wild-type $tRNA^{Asp}$ transcript. Both K_M and k_{cat} are strongly affected (150-fold each). A first reason that possibly explains this incomplete expression of aspartate identity concerns the unusual base pair G1·A72 present in the whole series of transcripts studied here. The first base pair within $tRNA^{Asp}$ corresponds to U1-A72 and is in direct contact with the synthetase as revealed by the crystal structure of the tRNA/synthetase complex (Cavarelli *et al.*, 1993). The synthetase tolerates also a G1·C72 sequence (Perret *et al.*, 1990a,b). In such a base pair chemical groups within the major groove, important for interaction with amino acids of the synthetase, are conserved. The presence of the non-Watson–Crick base pair in the acceptor stem may thus hinder optimal aspartylation. However, aminoacylation parameters of Asp^{Gln}_1 are poorly affected, and the K_M is increased about 3-fold. Thus, in the $tRNA^{Asp}$ context, the non-Watson–Crick first base pair G1·A72 does not modify significantly the positioning of the acceptor stem extremity within the yeast AspRS active site. The kinetic constants measured for Asp^{Gln}_2 and Asp^{Gln}_3 confirm also that the nucleotide sequence in the acceptor stem has no importance for the $tRNA^{Asp}$ recognition by yeast AspRS.

A second reason for the incomplete aspartylation of Gln^{Asp}_4 may be related to the absence of minor identity elements. Conversion of $tRNA^{Asp}$ into a glutamine acceptor tRNA leads to the observation that the sequence of nucleotide 38 influences aspartylation. Indeed, replacement of C38 by U38 decreases the affinity of the transcript and AspRS 5-fold and has a weak effect on the catalytic rate of the reaction. As a consequence U38 was replaced by C38 in Gln^{Asp}_4. This leads to a significant (590-fold) improvement in aspartylation efficiency. Thus, C38 is an additional identity nucleotide for yeast AspRS. Interestingly, the K_M effect measured between Asp^{Gln}_3 (C38) and Asp^{Gln}_4 (U38) (4.7-fold increase) is also observed between Gln^{Asp}_3 (U38) and Gln^{Asp}_4 (C38) (3.3-fold decrease). It seems that when C38 is present in Gln^{Asp}_4, affinity between the transcript and AspRS becomes sufficient to increase the complex half-life and then allows the optimal catalytic rate of the reaction to be reached. Analysis of the X-ray structure of the class II aspartate complex shows that positions N3 and N4 of C38 interact with the synthetase and should be important to constrain the tRNA structure during the complex formation. Interestingly, transplantation of the five aspartate identity elements as defined by Pütz et al. (1991) into the yeast $tRNA^{Phe}$ framework converted this tRNA into an aspartate acceptor only 12 times less efficient than wild-type $tRNA^{Asp}$ transcript. Indeed, yeast $tRNA^{Phe}$ possesses an A at position 38, a nucleotide presenting chemical groups (N3, N4) equivalent to those important in C38.

Changes at the level of the anticodon loop sequence of transcripts presenting the $tRNA^{Gln}$ framework lead to the discovery of an anticooperative relationship between glutamine identity elements. Indeed, variant Gln^{Asp}_3 has lost two major glutamine identity elements, nucleotides C34 and G36, and is no longer glutaminylatable. Further removal of an identity element, nucleotide U38 (variant Gln^{Asp}_4 where U38 is replaced by C38), leads to the recovery of a rather important glutaminylation capacity. The efficiency of the corresponding reaction is rather weak ($\mathcal{L}$ = 38 900-fold) as compared to that of wild-type $tRNA^{Gln}$ transcript but shows that large changes in the $tRNA^{Gln}$ anticodon sequence may have an anticooperative effect on the aminoacylation reaction. This is reminiscent of what was observed in the valine system represented by the turnip yellow mosaic virus tRNA-like domain and wheat germ ValRS (Dreher *et al.*, 1992) and in the yeast aspartate system (Pütz *et al.*, 1993), where anticooperative behaviors of multiple mutants of the RNAs were explained by the creation of novel interactions between the mutated anticodon loops and identity amino acids in the synthetases.

Effects of Different tRNA Scaffoldings on the Expression of Partial Identities. Two couples of transcripts present the same set of determinants belonging partially to aspartate and partially to glutamine identity sets. The first couple concerns Asp^{Gln}_5 and Gln^{Asp}_2, and the second one corresponds to Asp^{Gln}_6 and Gln^{Asp}_1 (Figure 4A,B). Interestingly, both variants of the same couple behave differently toward either synthetase (Table 1). Aspartylation of Gln^{Asp}_2 is not detectable ($\mathcal{L}$ >

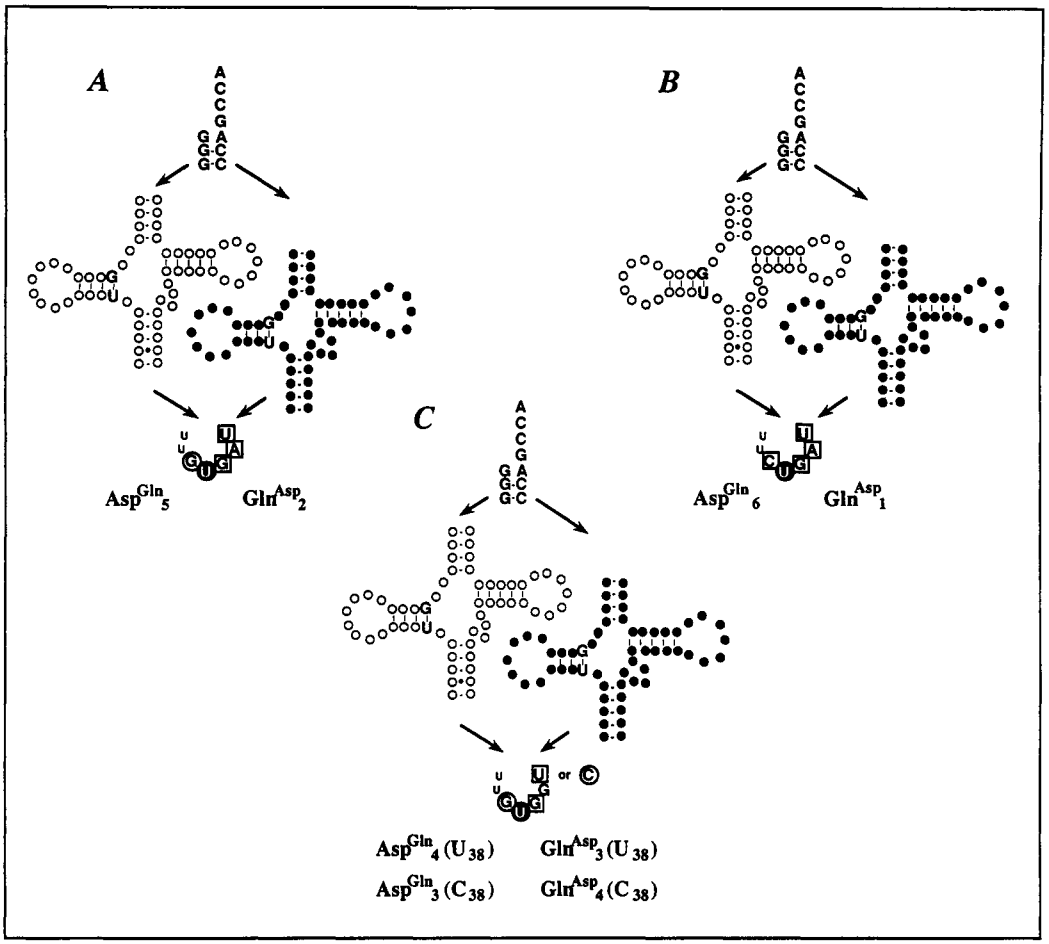

FIGURE 4: Schematized secondary structures of three families, A, B, and C, of variants presenting common features, in the aminoacyl-acceptor stem end, the anticodon loop, and base pair 10–25. Nucleotides within the anticodon loop belonging to the aspartate identity set are light circled, those belonging to the glutamine identity set are squared, and those belonging to both sets are dark circled. The aspartate framework is schematized by opened circles and the glutamine framework by dark circles. In panel C the anticodon loops of the variants present either U38 or C38 as highlighted in parentheses next to the names of the molecules.

10^6), whereas Asp^{Gln}_5 is still recognized by AspRS ($\mathcal{L} = 1400$). Conversely, Gln^{Asp}_2 is 5-fold more efficiently glutaminylated than Asp^{Gln}_5 ($\mathcal{L} = 35$ and $\mathcal{L} = 180$, respectively). Within the second couple, Asp^{Gln}_6 is aspartylated with a loss of efficiency of 6300 and Gln^{Asp}_1 with a loss of more than 10^6. Thus, one variant is at least 160 times better charged by AspRS than the other. Thus, aspartylation is expressed to a higher level in variants derived from host $tRNA^{Asp}$, and glutaminylation is expressed to a higher level in the variant derived from host $tRNA^{Gln}$.

This phenomenon can be further highlighted by the variability of effects brought by insertion or elimination of a given identity element toward the aminoacylation properties of the corresponding host tRNAs. As example, Asp^{Gln}_3 and Asp^{Gln}_4 (Figure 4C) differ only by the nature of nucleotide 38 in the anticodon loop. Asp^{Gln}_3 presents a cytidine and Asp^{Gln}_4 a uridine residue at this position. Replacement of C38 by U38 leads to a loss in aspartylation efficiency of a factor of 6. For variants Gln^{Asp}_4 and Gln^{Asp}_3 (Figure 4C) the presence of either C38 or U38 is correlated with a difference in aspartylation efficiency of a factor of 590. A similar situation appears when comparing, for example, the influence of position 36 on aspartylation. In the aspartate framework, replacement of C36 by G36 (Asp^{Gln}_4 and Asp^{Gln}_5, respectively) leads to a 66-fold decrease in aminoacylation efficiency, whereas in the glutamine framework, the same changes lead to a 531-fold loss in efficiency (Gln^{Asp}_3 and Gln^{Asp}_2). These examples highlight again that the level of expression of partial identity sets is directly correlated to the number of identity elements present as well as to the tRNA framework in which they are embedded.

As already discussed above, the structural frameworks of both $tRNA^{Asp}$ or $tRNA^{Gln}$ are not detrimental to the optimal expressions of the converse identities. Moreover, there are no negative elements hindering these expressions. Indeed, optimal or near-optimal aminoacylation efficiencies of the host tRNAs presenting complete novel identity sets could be obtained without additional changes at the level of the structural framework or of any other nucleotide not belonging to the identity set. Alternatively, the differential expressions of intermediate identities in either $tRNA^{Asp}$ or $tRNA^{Gln}$ frameworks may be explained by three nonexclusive reasons: (i) a nonoptimal plasticity of the host tRNA, limiting its conformational adaptation to the synthetase, (ii) transient exposure during this adaptation of elements percieved as negative signals by the synthetase, and (iii) an impossibility of cooperative relationships between identity elements, due to the absence of one or more of these elements.

Conformational Effects. Whereas transplantation of the complete glutamine identity set into the $tRNA^{Asp}$ framework led to an optimal glutaminylation of this chimeric tRNA, transplantation of the aspartate identity set, including elements defined by Pütz *et al.* (1991) and residue C38 as defined in this study, led only to suboptimal aspartylation ($\mathcal{L} = 32$ for Gln^{Asp}_4). As already suggested, this may be linked to the different plasticity of both $tRNA^{Asp}$ and host $tRNA^{Gln}$.

Indeed, the tertiary interaction (G10·U25)G45 was shown to have an indirect structural effect on aspartylation efficiency (Pütz *et al.*, 1991). Moreover, the relative orientation of the two branches of $tRNA^{Asp}$ is strongly modified when it is in contact with its synthetase (Ruff *et al.*, 1991), and consequently this structural motif, localized in the hinge region, is involved in the flexibility of $tRNA^{Asp}$ during the complex formation with AspRS. Two modifications are present within the variable loop of variant $Gln^{Asp}{}_5$ in order to allow the formation of a correct triple base pair between nucleotides 10–25–45, i.e., a $tRNA^{Asp}$-derived triple pair within the glutamine framework. These changes have, however, no incidence on the aspartylation properties of the transplanted $tRNA^{Gln}$. The converse experiments performed in the $tRNA^{Asp}$ framework, however, point to the great importance of base pair G10·U25 for aspartylation. Indeed, aspartylation of $Asp^{Gln}6$ containing only three aspartate identity elements (G10·U25, U35, and G73) is still possible, whereas $Asp^{Gln}{}_7$, where U25 is replaced by C25, is no longer recognized. Thus, as was the case for identity nucleotides in direct contact with the synthetase, subtile structural motifs are expressed differently according to the context.

Aminoacylation studies of yeast $tRNA^{Phe}$ derivatives transplanted with the aspartate identity set have shown that AspRS can tolerate some structural variabilities within its substrate (Perret *et al.*, 1992). Thus, suboptimal aspartylation of the glutamine-derived transcripts studied here is not necessarily related to direct structural constraints but, as suggested above, is rather linked to the exposure of antideterminants within the host tRNA bearing intermediate identity sets during the reciprocal adaptation of AspRS and its substrate.

The contribution of structural features on the glutaminylation of Asp^{Gln} variants was also tested. Integration of the complete set of $tRNA^{Gln}$ triple interactions ($Asp^{Gln}{}_8$) had a negative effect on the expression of the glutamine identity, suggesting that, in this case also, an optimal adaptation between substrate and enzyme is somewhat hindered, most probably by the appearance of negative signals during the transition state of the reaction. Interestingly and in contrast to these results, structural changes in a $tRNA^{Glu}$ containing the complete glutamine identity set lead to an improvement of glutaminylation ($\mathcal{L}$ varied from 90 to 25) (Rogers & Söll, 1993; Rogers *et al.*, 1993). Comparison of both results indicates that the structural framework of wild-type $tRNA^{Glu}$ has a negative effect and acts as an antideterminant for GlnRS recognition. This negative structural feature in $tRNA^{Glu}$ is very useful to decrease the mischarging capacities between these two closely related aminoacylation systems.

General Conclusions. We have shown here that simple transplantation of the complete set of glutamine identity nucleotides into $tRNA^{Asp}$ converts this tRNA to an optimal glutamine acceptor. Integration of the aspartate identity set, including the newly discovered identity element C38 into $tRNA^{Gln}$, leads also to an efficient, although suboptimal, transfer of specificity. These data clearly confirm that there are no bolts within tRNAs hindering cross-reactions with synthetases from the two classes or from both prokaryotic and eukaryotic kingdoms. A second main outcome of this study comes from the progressive character of the transplantation experiments performed. Indeed, progressive reciprocal transplantation of identity elements between both tRNAs leads to concomittant progressive gains in the new and losses in the native specificities. However, the levels of expression of a partial combination of identity elements vary significantly according to the host tRNA framework. A similar differential behavior is observed according to the framework when a single identity nucleotide is incorporated or removed. Our data suggest that the level of expression of a partial identity set within a noncognate host tRNA is dependent on the individual strength and the number of identity elements present. This number allows in turn a high degree of communication between the elements and their cooperative expression. The flexibility of the structural framework of the host tRNA, allowing or not allowing optimal adaptation between the tRNA and the synthetase, contributes as well.

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