Triple Aminoacylation Specificity of a Chimerized Transfer RNA†

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ABSTRACT: We report here the rational design and construction of a chimerized transfer RNA with tripartite aminoacylation specificity. A yeast aspartic acid specific tRNA was transformed into a highly efficient acceptor of alanine and phenylalanine and a moderate acceptor of valine. The transformation was guided by available knowledge of the requirements for aminoacylation by each of the three amino acids and was achieved by iterative changes in the local sequence context and the structural framework of the variable loop and the two variable regions of the dihydrouridine loop. The changes introduced to confer efficient acceptance of the three amino acids eliminate aminoacylation with aspartate. The interplay of determinants and antideterminants for different specific aminoacylations, and the constraints imposed by the structural framework, suggest that a tRNA with an appreciable capacity for more than three efficient aminoacylations may be inherently difficult to achieve.

Identity of tRNAs for their cognate aminoacyl-tRNA synthetases is governed by positive and negative elements that permit their specific aminoacylation while preventing false charging (Normanly & Abelson, 1989; Schimmel, 1989; Söll, 1991; Giegé et al., 1993). The positive elements, or identity determinants, are a limited number of nucleotides located at strategic positions on the surface of the L-shaped RNA scaffolding. These elements have been found in several instances in contact with amino acids of the synthetase (Rould et al., 1989; Ruff et al., 1991; Rudinger et al., 1992; Cavarelli et al., 1993), although indirect effects are not excluded. It is likely that these specific interactions activate the catalytic site of the enzymes and thus trigger aminoacylation. Specific complex formation is accompanied by conformational changes [e.g., Lam and Schimmel (1975), Krauss et al. (1976), Riesner et al. (1976), and Ruff et al. (1991)] which are partly lost in the tRNA upon mutation of identity positions (Rudinger et al., 1992). The negative elements, or identity antideterminants, are structural features that prevent a tRNA from being recognized and aminoacylated by noncognate aminoacyl-tRNA synthetases (Muramatsu et al., 1988; Perret et al., 1990a).

Identity nucleotides have been defined for a large number of systems by either *in vitro* or *in vivo* techniques [e.g., Normanly et al. (1986), Hou and Schimmel (1988), McClain and Foss (1988a), and Sampson and Uhlenbeck (1988); reviewed in Schulman (1991), Pallanck and Schulman (1992), Giegé et al. (1993), and McClain (1993)]. They are mainly located in three parts of the tRNA structure, as seen in Figure 1, which are the discriminator position N73 as well as the neighboring helical region of the amino acid accepting stem, the anticodon arm, and the D-arm. In a few cases specific chemical groups could be characterized as the identity signals, either by crystallographic (Rould et al., 1991; Cavarelli et al., 1993) or RNA engineering methods (Musier-Forsyth et al., 1991; Musier-Forsyth & Schimmel, 1992).

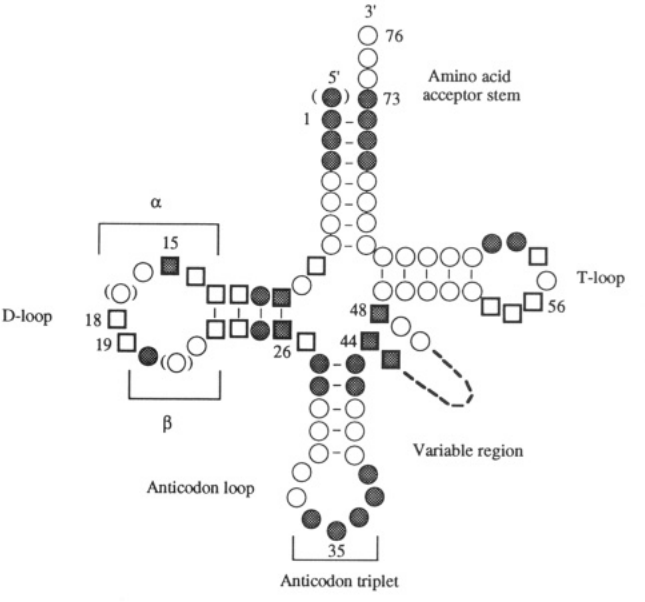

FIGURE 1: Cloverleaf structure of tRNAs with the locations of major identity nucleotides, summarizing the present knowledge on the location of identity nucleotides revealed by both *in vivo* and *in vitro* methods (for references, see the text). Identity positions are shaded. Positions involved in the higher order structure of tRNA are indicated by boxes. Numbering of residues is according to Steinberg et al. (1993). Notice the variable length of the D-loop (α- and β-regions of 2–4 residues).

The concept of identity implies two main consequences. First, transplantation of identity elements from one tRNA context into another should confer to the engineered molecules a new specificity. This has been confirmed in transplantation experiments, which were widely used to assess the completeness of identity sets defined by mutational analyses, although

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optimal aminoacylation activities within a new context were not always achieved [e.g., Schulman and Pelka (1988), Sampson et al. (1989), Pütz et al. (1991), McClain and Foss (1988b), Rogers and Söll (1988), and Rogers et al. (1992)]. Second, because of the rather widespread location of identity positions in tRNAs, it should be conceptually possible to create artificial tRNAs with multiple charging abilities. The possibility of designing such molecules is strengthened by the observation that either canonical or suppressor species can be mischarged *in vitro* and *in vivo* [e.g., Jacobson (1971), Shimura et al. (1972), Roe et al. (1973), Yarus and Mertes (1973), Giegé et al. (1974, 1993), Normanly and Abelson (1986), Hou and Schimmel (1988, 1989b), Schimmel (1990), Pallanck and Schulman (1991), McClain et al. (1991), and Pallanck et al. (1992)]. Indeed, engineered suppressor tRNAs were shown to be charged by several aminoacyl-tRNA synthetases because different amino acids were found incorporated at the same internal stop codon. Additionally, overexpression of synthetases can lead to significant mischarging in the cellular context (Sherman et al., 1992a,b).

The existence of various effects modulating the expression of tRNA identities has been described in several systems. For instance, minor identity nucleotides have been found for both $tRNA^{Phe}$ (Sampson et al., 1992) and *Escherichia coli* $tRNA^{Ala}$ (Shi et al., 1990; McClain et al., 1991; Francklyn et al., 1992b). For alanine identity, systematic analysis of the aminoacylation capacity of minihelices pointed to a sequence context requirement for optimal expression of alanylation. The presence of a G2·C71 base pair stacked over the major identity base pair G3·U70 is important for alanylation of minihelices (Francklyn et al., 1992b). This also holds true for the complete $tRNA^{Ala}$ (McClain et al., 1991). Alanylation has also been shown to be influenced by the discriminator base A73 (Shi et al., 1990; McClain et al., 1991; Shi & Schimmel, 1991; Francklyn et al., 1992b). From another point of view, conformational effects modulating aminoacylation identities were described for the transplantation of the phenylalanine identity within the yeast $tRNA^{Asp}$ framework (Perret et al., 1992). Here enhanced phenylalanylation activities were obtained by changing the D-loop and variable region structures. Involvement of structural effects was also stressed for an optimal expression of the alanine identity (McClain et al., 1991), and in the case of cysteine identity, an unusual Levit base pair plays a major role (Hou et al., 1993). Finally, expression of an identity set can be completely shielded by an antideterminant, as exemplified in yeast $tRNA^{Asp}$ which is not arginylated because of the presence of the modified nucleotides (Perret et al., 1990a). As a consequence, optimal activity of multiple aminoacylation specificities within a tRNA should be dependent on the individual requirements for the expression of each specificity, and these requirements may not be the same. Additionally, positive signals for one synthetase may be negative signals for the other synthetase.

Following these lines, the primary purpose of the experiments described in this paper was to create by rational design a novel tRNA with efficient multipartite aminoacylation identity. However, attaining this goal may be difficult, because expression of identities, as occurs in biological systems selected by evolution, is more complex than would be expected from the simplified view discussed above. Indeed, expression of additional specificities in tRNAs, as observed in mischarging experiments, is often poor even in the more recent experiments using engineered suppressor tRNAs. This is likely the consequence of the incompleteness of the identity sets corresponding to the mischarged amino acids, but could also be related to the absence of minor identity elements (Shi et al., 1990; McClain et al., 1991) or of discrete elements contributing to tuning the structural framework of the tRNA (McClain et al., 1991; Perret et al., 1992) or to the presence of antideterminants (Muramatsu et al., 1988; Perret et al., 1990a). An example of a tRNA with an *in vitro* double specificity that has been studied in some detail is the *E. coli* $tRNA^{Tyr}$ amber suppressor with the major alanine identity base pair G3·U70 introduced into the acceptor stem (Hou & Schimmel, 1989b). The G3·U70 $tRNA^{Tyr}$ amber suppressor remains an efficient substrate for TyrRS[1] and is an efficient substrate for *E. coli* AlaRS. The optimal expression of both activities in this tRNA can be explained by the presence in the $tRNA^{Tyr}$ context of the minor alanine identity elements A73 and G2·C71 that contribute to better alanylation.

Taking into account all these considerations, we were able to design a chimeric tRNA with multiple optimal aminoacylation specificities, thus bypassing the constraints introduced by evolution in wild-type molecules to ensure their *in vivo* specificity. To attain this goal, but also to test globally in the same series of experiments the involvement of structural and contextual effects on identity, we have deliberately chosen a model system in which the host tRNA, yeast $tRNA^{Asp}$, possesses a conformation different from that of the tRNAs donating their identity sets. These tRNAs were yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Ala}$. Their deviations from the conformation of $tRNA^{Asp}$ are the consequence of sequence differences in the D-loop and the variable loop as seen in Figure 2. For $tRNA^{Asp}$ and $tRNA^{Phe}$ the differences are confirmed by crystallographic (Rich & RajBhandary, 1976; Westhof et al., 1985) and solution data (Romby et al., 1985, 1987). As a favorable prerequisite for the purpose of this work, $tRNA^{Phe}$ and $tRNA^{Ala}$ have completely disconnected major identity sets: A73, G20, G34, A35, and A36 for yeast $tRNA^{Phe}$ (Sampson et al., 1989) and base pair G3·U70 for *E. coli* $tRNA^{Ala}$ (Hou & Schimmel, 1988; McClain et al., 1988a).

Considering all these features, our starting molecule (called Asp^{AF}_1)[2] for engineering a chimeric tRNA has basically the yeast $tRNA^{Asp}$ sequence and conformation with the phenylalanine and alanine identity sets (Figure 3). In this molecule only two aspartate identity elements (Pütz et al., 1991) (base pair G10·U25 and nucleotide G34) are conserved, the three others (U35, C36, G73) being replaced by phenylalanine identity nucleotides. As expected from the previous considerations, it was found that the expression of the alanine and phenylalanine specificities is not optimal in this molecule but can be enhanced and optimized by manipulating structure and sequence context. These molecules have lost their aspartylation ability but have become additionally moderate valine acceptors. The implications of these results as well as perspectives for *in vitro* applications are considered.

MATERIALS AND METHODS

Materials. Oligonucleotides 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 (23 Ci/mol), L-[^{3}H]phenylalanine (26 Ci/mol), L-[^{3}H]valine (30 Ci/mol), and L-[^{3}H]alanine (58

[1] Abbreviations: AlaRS, AspRS, PheRS, TyrRS, and ValRS, alanyl-, aspartyl-, phenylalanyl-, tyrosyl-, and valyl-tRNA synthetase, respectively.

[2] Notation: Asp, the basic sequence and structure is from yeast $tRNA^{Asp}$; AF, it possesses the alanine (A) and phenylalanine (F) major identity nucleotides; 1, the first variant of a series.

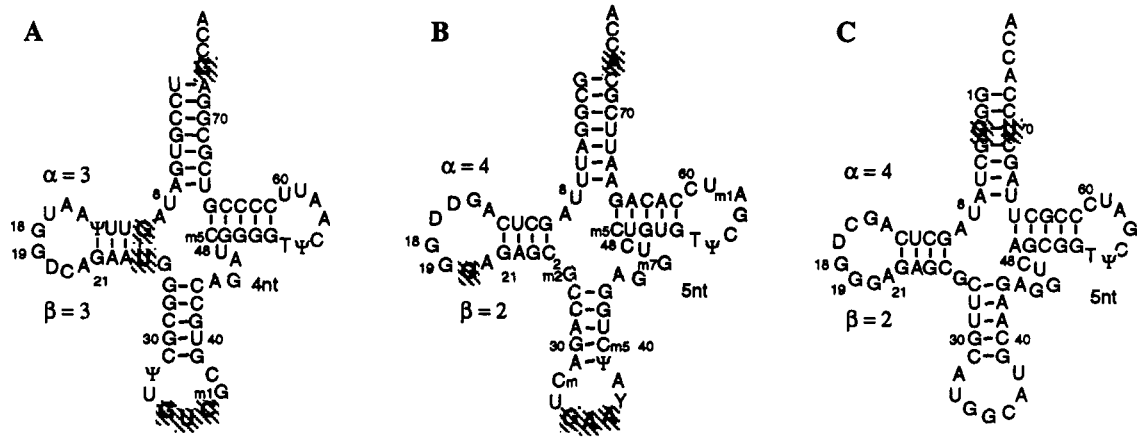

FIGURE 2: Cloverleaf structures of yeast $tRNA^{Asp}$ (A), yeast $tRNA^{Phe}$ (B), and *E. coli* $tRNA^{Ala}$ (C). The major identity nucleotides have been highlighted. The lengths of regions α and β lying beside the conserved G18-G19 sequences in the D-loops are mentioned, as are the length of the variable loops.

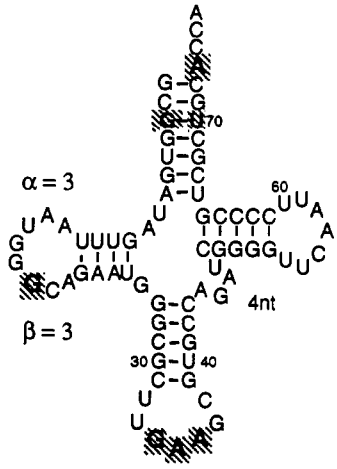

FIGURE 3: Cloverleaf structure of the chimerized $tRNA^{Asp}$ sequence Asp^{AF_1} containing the major identity nucleotides specifying phenylalanylation by yeast PheRS and alanylation by *E. coli* AlaRS (shaded).

Ci/mol) were from Amersham France (Les Ulis). *E. coli* AlaRS (Regan et al., 1986; Hill & Schimmel, 1989), yeast AspRS (Lorber et al., 1983), yeast ValRS (Kern et al., 1975), and T7 RNA polymerase (Wyatt et al., 1991) were purified as described previously. Pure yeast PheRS was a kind gift of M. Baltzinger (Strasbourg). A yeast homogenate was used as a crude preparation of synthetases. Restriction enzymes *Bst*NI, *Hin*dIII, and *Bam*HI and T4 polynucleotide kinase were from New England Biolabs (Beverly, MA). T4 DNA ligase was from Boehringer.

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 genes were constructed by hybridization and ligation of a series of overlapping oligonucleotides and cloning into pUC 119 linearized at *Bam*HI and *Hin*dIII sites. *E. coli* Tg1 cells were transformed. A *Bst*NI site juxtaposed to the 3'-end of the tRNA sequences allowed synthesis of tRNAs ending with the expected CCA sequence. *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 dithiothreitol, 0.01% Triton X100, 1 mM spermidine, 4 mM each nucleoside triphosphate, 5 mM GMP, 0.1 $\mu g/\mu L$ linearized plasmid, and an adequate amount of T7 RNA polymerase. Incubations were for 3 h at 37 °C, and reactions were stopped by phenol/chloroform extraction. Since T7 RNA polymerase makes some errors at the 3'-end of the transcripts, full-length transcripts correctly ending with the CCA sequence have been purified by electrophoresis on 12% polyacrylamide denaturing gels followed by electroelution. Concentrations of stock solutions of transcripts have been determined by absorbance measurements at 260 nm.

Aminoacylation Reactions. All aminoacylation reactions have been performed in the same medium containing 25 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 0.5 mM ATP, 0.1 mg/mL bovine serum albumin, 50 μM ^{3}H-labeled amino acid, and appropriate amounts of tRNA transcript and synthetase. Transcripts have been renatured before aminoacylation by heating to 65 °C for 90 s and slow cooling to room temperature. Incubations were at 30 °C, and aminoacylated tRNA samples were quenched and treated in the conventional way (Perret et al., 1990a). Plateau levels of charging were established in the presence of 1 μM transcripts and 85 nM pure synthetase (or appropriate amounts of crude synthetase preparation). The kinetic constants were derived from Lineweaver–Burk plots. They represent an average of two independent experiments.

RESULTS

Aminoacylation Capacities of a tRNA with Two Distinct Identity Sets. The ability of *E. coli* AlaRS, yeast AspRS, and yeast PheRS to recognize and charge the chimeric Asp^{AF_1} $tRNA^{Asp}$ mutant has been investigated. The alanylation and phenylalanylation efficiencies of transcript Asp^{AF_1} were studied and compared to those of wild-type transcripts of yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Ala}$, respectively. Identical aminoacylation conditions have been chosen to test both phenylalanylation and alanylation. A time course of these aminoacylations is shown in Figure 4, and the steady-state kinetic parameters of these reactions are presented in Table I. As expected, the chimeric transcript Asp^{AF_1} was recognized and charged by both enzymes, as revealed first by the levels of aminoacylation. However, these levels are rather low as compared to the corresponding aminoacylations of wild-type transcripts. Low efficiency in alanylation and phenylalanylation is also shown by low k_{cat} and high K_M values. Alanylation of transcript Asp^{AF_1} is about 8850 times less efficient than is alanylation of $tRNA^{Ala}$ transcript, and its phenylalanylation is 285 times less efficient than the corresponding control. Thus, although these results show that it is possible to create a tRNA with multiple specificities by simple transplantation of several identity sets into a common host tRNA, the expression of these sets is clearly not optimal. Also, the complete lack of

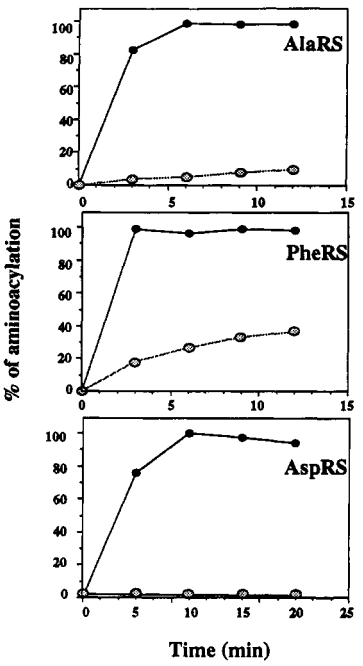

FIGURE 4: Kinetics of transcript Asp^{AF}_1 aminoacylation catalyzed by pure synthetases (stippled ovals). Aminoacylation levels are normalized relative to the charging of the homologous wild-type yeast $tRNA^{Phe}$, *E. coli* $tRNA^{Ala}$, and yeast $tRNA^{Asp}$ transcripts, respectively (solid ovals).

Table I: Kinetic Parameters for Aminoacylation of Several Transcripts Derived from Yeast $tRNA^{Asp}$ with Yeast PheRS and *E. coli* AlaRS[a]

tRNA transcripts	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M	k_{cat}/K_M (rel)	L
Alanylation					
wild-type transcript and double transplant					
Ala	163	0.245	665	1	1
Asp^{AF}_1	2	26.5	0.075	0.000 113	8850
structural variants					
Asp^{AF}_3	10	19.3	0.518	0.000 779	1280
Asp^{AF}_5	5	26.5	0.188	0.000 282	3570
sequence variant					
Asp^{AF}_2	162	1.625	99.7	0.149	7
structural and sequence variants					
Asp^{AF}_4	256	0.325	787	1.18	0.8
Asp^{AF}_6	313	1	313	0.47	2.1
Phenylalanylation					
wild-type transcript and double transplant					
Phe	120	0.300	400	1	1
Asp^{AF}_1	3	2.185	1.37	0.0035	285
structural variants					
Asp^{AF}_3	7	0.710	9.9	0.024	42
Asp^{AF}_5	7	0.545	12.8	0.032	31
sequence variant					
Asp^{AF}_2	19	0.205	9.27	0.23	4.3
structural and sequence variants					
Asp^{AF}_4	59	0.215	274	0.686	1.45
Asp^{AF}_6	30	0.105	286	0.715	1.4

[a] Aminoacylation conditions were the same for both enzymes (see Materials and Methods). Apparent K_M and k_{cat} values for each $tRNA^{Asp}$ variant were derived from Lineweaver–Burk plots. The relative k_{cat}/K_M values varied by 30–50%. L corresponds to the loss in catalytic efficiency (inverse of the relative k_{cat}/K_M). Names of mutants are relative to Figure 5.

aspartylation of transcript Asp^{AF}_1 (Figure 4), although it still contains two aspartate determinants (G34 and the G10·U25 base pair), is noticeable.

Aminoacylation of Structural Mutants. In order to test the influence of the structural framework on the expression of the phenylalanine and alanine identity sets in $tRNA^{Asp}$, two new transcripts, derived from Asp^{AF}_1, have been created (Figure 5). Asp^{AF}_3 has a D-loop with $\alpha = 4$ and $\beta = 2$ regions. This has been achieved by insertion of a uridine residue (common to $tRNA^{Phe}$ and $tRNA^{Ala}$) at the 3′-side of G18 and deletion of residue C20:1 in the β-region of the D-loop. Variant Asp^{AF}_5 shows the structural characteristics of Asp^{AF}_3, but in addition has a variable loop of five nucleotides identical in sequence to both $tRNA^{Phe}$ and $tRNA^{Ala}$. Structural changes in this kind of variant compared to wild type have been established previously by lead cleavage analysis (Perret et al., 1992). The kinetic characteristics of the aminoacylations of both of these variants are presented in Table I.

As expected, the phenylalanylation efficiencies of both structural mutants derived from Asp^{AF}_1 are improved by factors of 7–9. The catalytic rate constant k_{cat} was slightly increased, and K_M decreased 3–4-fold, for both mutants. Thus, mutant Asp^{AF3} with fine structural changes in the D-loop remains only 42 times less efficient, and mutant Asp^{AF}_5 with structural changes in both the D-loop and the variable loop remains only 31 times less efficient than wild-type $tRNA^{Phe}$, while Asp^{AF}_1 was about 285 times less efficient. These results confirm the importance of the structural context for the expression of phenylalanine identity.

Alanylation is also sensitive to the structural framework in which the identity base pair G3·U70 is embedded. Indeed, variants Asp^{AF}_3 and Asp^{AF}_5 both show improved levels of charging and improved kinetic parameters (Table I). Remodeling the structural characteristics of the D-loop alone (Asp^{AF}_3) ended up with a loss in alanylation of more than 1200-fold; this corresponds to an improvement of about 7 times compared to Asp^{AF}_1. Remodeling of the structural characteristics of both the D-loop and the variable loop (Asp^{AF}_5) led also to an improvement of alanylation. However, this improvement (2.5-fold) is not as important as in the case of variant Asp^{AF3}.

Aminoacylation of Sequence Mutants. Variant Asp^{AF2} is derived from Asp^{AF}_1 by single conversion of base pair C2·G71 to G2·C71 (Figure 5). The alanylation of this transcript was markedly improved at both the k_{cat} and K_M levels compared to that of transcript Asp^{AF1} (Table I). The aminoacylation efficiency, k_{cat}/K_M, is considerably increased and is only 7 times lower than that of the wild-type $tRNA^{Ala}$. This corresponds to an improvement of alanylation compared to that of Asp^{AF}_1 of more than 1200-fold. Although this kind of effect could be predicted for alanylation, no effect of base pair G2·C71 was expected on phenylalanylation because this base pair was deduced not to be important for phenylalanine identity (Sampson et al., 1989). However, results presented in Table I clearly demonstrate that this sequence is very important for the expression of the phenylalanine identity in the $tRNA^{Asp}$ context. Indeed, k_{cat} was improved 2–3 times, and K_M was lowered 10 times, compared to those of Asp^{AF}_1. Thus, Asp^{AF}_2, is only 4–5 times less efficiently phenylalanylated than the $tRNA^{Phe}$ transcript. Incorporation of base pair G2·C71 improves the phenylalanylation of Asp^{AF}_1 by as much as 66 times.

Aminoacylation of tRNAs with Changes in Sequence and Structure. Two transcripts of $tRNA^{Asp}$, possessing in addition to the major identity nucleotides responsible for alanine and phenylalanine specificities a combination of sequence and structure contexts characteristic of $tRNA^{Phe}$ and $tRNA^{Ala}$, have been created. Asp^{AF}_4 presents base pair G2·C71 and

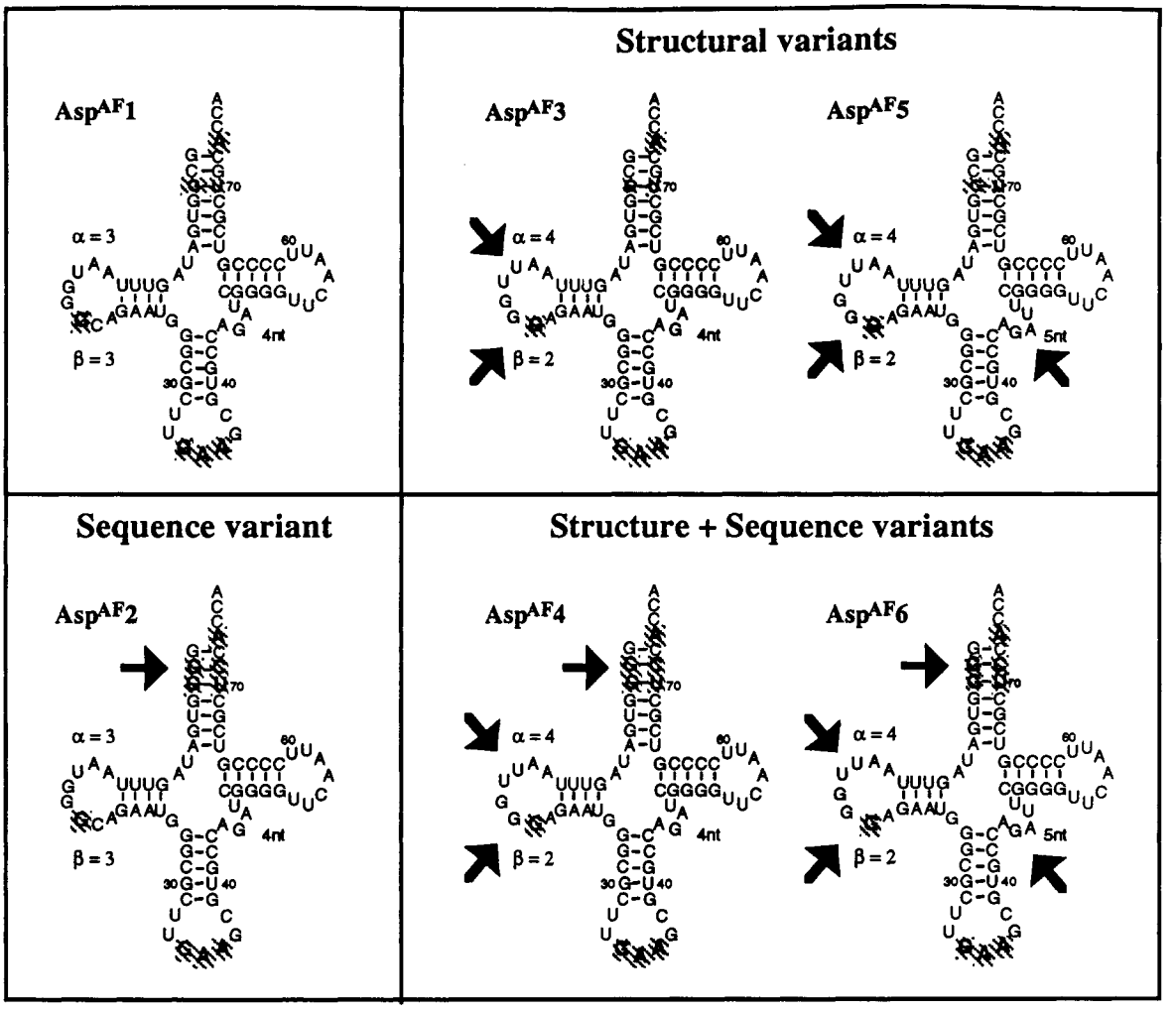

FIGURE 5: Cloverleaf sequences of $tRNA^{Asp}$ variant transcripts. Asp^{AF_3} and Asp^{AF_5} present alterations in the D-loop and/or the variable loop compared to Asp^{AF_1}; Asp^{AF_2} is structurally identical to Asp^{AF_1} but shows a sequence alteration at base pair 2·71; Asp^{AF_5} and Asp^{AF_6} present both structural changes at the level of the D-loop and/or the variable loop and the sequence change at base pair 2·71 compared to Asp^{AF_1}. Arrows highlight the mutated domains. In contrast to the natural $tRNA^{Asp}$, all transcripts contain a G1·C72 base pair for efficient transcription (Perret et al., 1990b) and are deprived of modified bases.

changes in the D-loop, whereas Asp^{AF_6} presents in addition to the changes already discussed for Asp^{AF_4} a variable loop of five nucleotides (Figure 5). In other words, Asp^{AF_4} is a combination of Asp^{AF_2} and Asp^{AF_3}, and Asp^{AF_6} is a combination of Asp^{AF_2} and Asp^{AF_5}. The kinetic characteristics of these two new variants, displayed in Table I, point to the optimal expression of both identity sets. Indeed, the levels of alanylation of these transcripts are very high and similar to those of the $tRNA^{Ala}$ transcript; k_{cat} values are up to 2-fold higher than, and K_M values are within 4-fold of, those of wild-type $tRNA^{Ala}$. Finally, overall alanylation efficiency of Asp^{AF_4} reaches that of the wild type and that of Asp^{AF_6} remains only 2 times less efficient.

Phenylalanylation of these transcripts appears also to be efficient because k_{cat} values remain only 2–4-fold lower than for wild type, and K_M values are also 2–3-fold lower, suggesting a better affinity for the synthetase than is shown by wild type $tRNA^{Phe}$ transcript. Finally, k_{cat}/K_M for these transcripts is about the same as for wild-type (Table I). Thus, engineering of both the structural context and the sequence neighborhood in the amino acid acceptor stem in yeast $tRNA^{Asp}$ transcript, into which identity sets specific for both phenylalanylation and alanylation have been integrated, leads to a tRNA with quasi-optimal aminoacylation activities toward both yeast PheRS and *E. coli* AlaRS.

Aspartylation and Valylation Properties of $tRNA^{Asp}$ Chimeras. All six $tRNA^{Asp}$ mutants studied here have lost several identity nucleotides responsible for aspartylation, namely, the discriminator base G73 and nucleotides U35 and C36 of the anticodon. They present, however, the G10·U25 base pair, as well as nucleotide G34, which together form the rest of the identity set. Thus, they may all present some residual aspartylation activity. Plateau levels of aspartylation of these mutants reached at most about 0.2% (not shown), suggesting that the residual aspartate identity nucleotides are not sufficiently strong to maintain clear aspartylation of the variant tRNAs. This result is corroborated by the unmeasurable residual aspartylation efficiency of a triple mutant of $tRNA^{Asp}$ where positions G73, U35, and C36 have been replaced by A73, A35, and A36 (Pütz et al., 1993).

Variant Asp^{AF_1} may contain partial identity sets responsible for specificities other than alanine and phenylalanine. Indeed, it contains both nucleotides A73 and A35, which correspond to major identity nucleotides responsible for valylation by ValRS from *E.coli* (Schulman & Pelka, 1985; Tamura et al., 1991b), yeast (Florentz et al., 1991), and wheat germ (Dreher et al., 1992). Indeed, this transcript appears to be an efficient substrate for yeast ValRS with a valylation efficiency only about 200 times lower than that of the wild-type $tRNA^{Val}$ transcript (Table II). The best yeast tRNAs mischarged by

Table II: Kinetic Parameters for Valylation of Several Transcripts Derived from Yeast $tRNA^{Asp}$ with Yeast ValRS[a]

tRNA transcripts	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M	k_{cat}/K_M (rel)	L
wild-type transcript and double transplant					
Val	74	0.080	925	1	1
$Asp^{AF}{}_1$	0.37	0.095	3.9	0.0042	238
structural variants					
$Asp^{AF}{}_3$	0.76	0.115	6.6	0.007	143
$Asp^{AF}{}_5$	2.5	0.16	15.6	0.017	59
sequence variants					
$Asp^{AF}{}_2$	0.70	0.11	6.3	0.0068	147
structural and sequence variants					
$Asp^{AF}{}_4$	0.82	0.18	4.6	0.0049	204
$Asp^{AF}{}_6$	1.30	0.15	8.6	0.0093	107

[a] L-values correspond to the loss in catalytic efficiency (inverse of the relative k_{cat}/K_M). Other conditions were as in Table I.

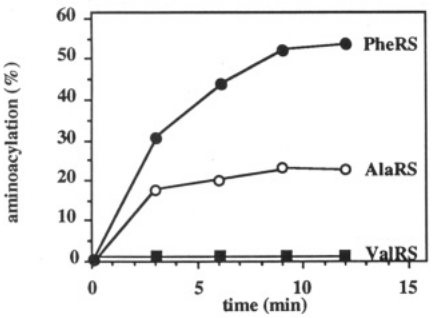

FIGURE 6: Aminoacylation of the engineered chimeric $tRNA^{Asp}$ $Asp^{AF}{}_4$ transcript with a crude yeast extract. Aminoacylation assays for the three activities were conducted in the presence of either alanine, phenylalanine, or valine and the same amount of crude extract.

yeast ValRS show losses in efficiency of at least 10 000-fold (Kern et al., 1972; Giegé et al., 1993), clearly demonstrating the efficiency of valylation of the chimeric $tRNA^{Asp}$. The other structural or sequence variants of $Asp^{AF}{}_1$ appeared to be even somewhat improved substrates for yeast ValRS, with $Asp^{AF}{}_5$ being about 4 times more efficient than $Asp^{AF}{}_4$. The charging levels of the whole series of mutants are rather high, the K_M's being unchanged and the k_{cat}'s only 30–200 times lower than for the wild-type $tRNA^{Val}$ transcript. These results suggest that major valine identity elements are efficiently expressed in the $tRNA^{Asp}$ context and that ValRS is not very sensitive to structural features of the D-loop and the variable loop or to the nature of base pair 2·71. They suggest also that the lacking valine identity elements are not compensated by a combination of alternative residues present in the $tRNA^{Asp}$ structure.

Multiple Aminoacylations under Conditions Mimicking in Vivo Conditions. The aminoacylation properties of $tRNA^{Asp}$ transcript $Asp^{AF}{}_4$ have been tested in the presence of a crude extract of yeast cells containing the active synthetases in independent experiments for each amino acid specificity. This transcript is charged to plateau levels of 25% with alanine and of 55% with phenylalanine, but no detectable charging with valine could be seen (Figure 6). This shows that, under conditions mimicking the *in vivo* situation, a tRNA with multiple specificities can be recognized as an active substrate by several synthetases. There is competition between AlaRS, PheRS, and ValRS for the same tRNA substrate; whereas both AlaRS and PheRS compete efficiently and equally, ValRS cannot be expressed, apparently because the two former synthetases completely sequester the chimeric tRNA [cf. Hou and Schimmel (1989b)]. The charging of the chimera by

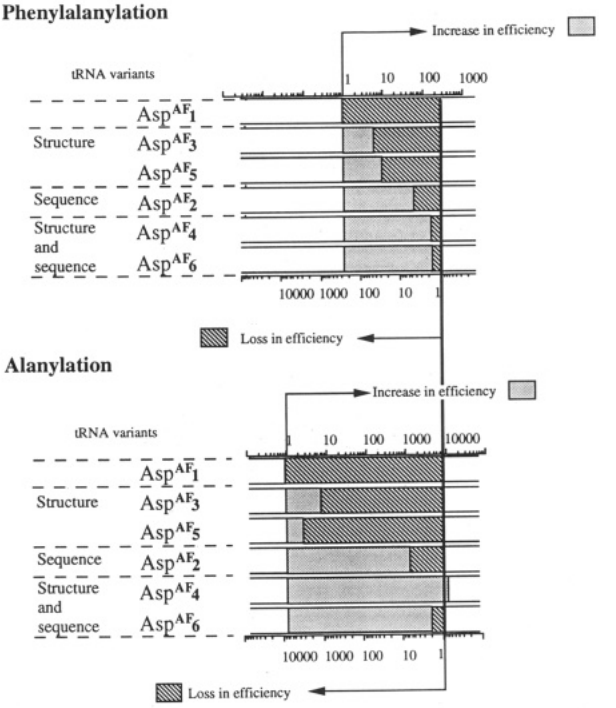

FIGURE 7: Schematic representation of the effects on aminoacylation of structural and/or sequence changes in a chimeric $tRNA^{Asp}$ possessing the major phenylalanine and alanine identity nucleotides. Hatched boxes emphasize losses in efficiency, while gray boxes represent stimulations in aminoacylation activity. The data are from Table I, and the scales are logarithmic. The reference transcript for calculating increase in catalytic efficiency is $Asp^{AF}{}_1$; for calculating loss in efficiency it is wild-type $tRNA^{Ala}$ or $tRNA^{Phe}$ transcript (see Table I) for alanylation or phenylalanylation activities, respectively. The wild-type-level activities are depicted by the bold vertical line at the right.

both alanine and phenylalanine was further established in experiments where the two amino acids were present, one labeled and one unlabeled. Both combinations were tested. Under those conditions, the plateau levels and initial rates with the labeled amino acid were reduced (not shown).

It is interesting to notice the high levels of charging of the chimera by alanine and phenylalanine with the crude extract, which compares well with the aminoacylation plateaus observed with pure enzymes (e.g., about 25% and 55% with crude extract compared with 80% and 90% with pure AlaRS and PheRS, respectively). When aminoacylation is done in the presence of both amino acids, the total amount of charged chimera levels at about 65%.

DISCUSSION

The results of this study show that the presence of identity nucleotides within a tRNA framework is not the sole requirement to allow optimal expression of aminoacylation specificities. They indicate that the simple view of identity expression and its implication for transplantation have to be refined. In what follows, we will discuss (i) the role of the structural framework of tRNA for the expression of identity elements, (ii) the influence of sequence context, and (iii) the combined effects of both conformation and sequence context. The experimental data are summarized in Figure 7.

Structural Framework. Because tRNAs fulfill common functions when passing through the protein-synthesizing machinery, it is believed that they all possess the same tertiary structure, a fact well supported by the existence of conserved and semiconserved residues that account for this structure. Therefore, less attention was paid to subtle conformational

differences between various tRNAs that could play a role in specific functions of these molecules, in particular, for their aminoacylations. Using yeast $tRNA^{Asp}$ as the model molecule to study identity set transplantations could allow an approach to the question of the relationship between conformation and activity, because of the peculiar structural properties of this tRNA, which differentiate it from most other species, in particular, from *E. coli* $tRNA^{Ala}$, yeast $tRNA^{Phe}$, and $tRNA^{Val}$. These differences rely on sequence variabilities in the D-loops and the variable regions (see Figure 2).

As expected from the concept of identity, transplantation of the alanine and phenylalanine identity sets directly into $tRNA^{Asp}$ confers to the engineered molecule the new specificities, but as was also anticipated, the expression of the novel identities is poor. Indeed, compared to the wild-type counterpart, phenylalanylation efficiency is reduced about 285 times, and alanylation, up to 8850 times (Figure 7). In agreement with our expectations, changing the conformational context of the basic $tRNA^{Asp}$ into a structure closer to that of $tRNA^{Phe}$ and $tRNA^{Ala}$ improves the expression of the phenylalanine and alanine identities.

The importance on phenylalanylation by yeast PheRS of the structural framework in which identity nucleotides are embedded, and in particular the D-loop and variable loop arrangements, has been demonstrated previously (Perret et al., 1992). Here again, in the presence of the phenylalanine and alanine identity nucleotides, changing smoothly the structure of the D-loop and the variable loop of $tRNA^{Asp}$ allows an increased phenylalanylation of the Asp^{AF} variants of about 7–9-fold. By modifying the sequence arrangement of the conserved residues in the D-loop, and thus their relative orientation to T-loop residues, and by increasing the length of the variable region, we imagined that novel tRNA conformers would be generated that better adapt to PheRS. In particular, the variable pocket conformation (Ladner et al., 1975) formed by the nonconserved residues 16, 17, and 20 in the D-loop and 59 and 60 in the T-loop would be different in $tRNA^{Asp}$ and $tRNA^{Phe}$ because it is governed by the relative positioning of the α- and β-regions around conserved residues G18 and G19 (see Figure 1). This interpretation is further supported by crystallographic studies on wild-type $tRNA^{Phe}$ [reviewed in Rich and RajBhandary (1976)] and $tRNA^{Asp}$ (Westhof et al., 1985), as well as by comparative solution studies of both tRNA structures with chemical probes (Romby et al., 1985, 1987), that have shown a large contribution of the D-loop and variable loop conformations to the overall tRNA structures. However, the weakness of the functional improvements brought by the structural changes in the D-loop and the variable region was unexpected, especially because they have brought one identity element of phenylalanine, namely, residue G20 located within the engineered D-loop region, into a more $tRNA^{Phe}$-like conformational environment. Thus, the structural changes introduced into the chimeric Asp^{AF}_5 molecules that were expected to influence the relative positioning of the anticodon and CCA regions and to allow a better presentation and/or adaptation of the chimeric tRNA to PheRS are not sufficient to trigger optimal phenylalanylation.

Alanylation with *E. coli* AlaRS of structural mutants Asp^{AF}_3 and Asp^{AF}_5 also becomes 2.5–7 times more efficient than alanylation of Asp^{AF}_1 (see Figure 7). The involvement of a conformational feature, namely, the variable pocket formed by nucleotides 16, 17, 20, 59, and 60 (Ladner et al., 1975), for alanylation of $tRNA^{Ala}$ *in vivo* was already shown (McClain et al., 1991). This variable pocket brings three residues from the D-loop, including G20, into close proximity to two residues from the T-loop, and it was explicitly shown that residue G20 contributes to the alanine identity (McClain et al., 1991; Tamura et al., 1991a). Because the wild-type sequence of $tRNA^{Asp}$ (see Figure 1) contains an additional nucleotide in the β-domain of the D-loop, it is conceivable that the resulting conformation will perturb the productive interaction with AlaRS by causing steric or ionic clashes, as was proposed earlier for nonoptimal transplantations of alanine identity sets in various tRNA contexts (McClain et al., 1991). Alternatively, the additional residue may cause conformational changes locally or at a distance within the tRNA that hinder alanylation activity. However, and in contrast with the phenylalanine system, it is understandable that the global structure is not of major importance in the alanine system since minihelices, corresponding to the sole acceptor domain of $tRNA^{Ala}$, were shown to be efficiently alanylated [reviewed in Francklyn et al. (1992a) and Musier-Forsyth and Schimmel (1993)].

Sequence Context and/or Minor Identity Nucleotides. In several systems, aminoacylation is driven not only by the major identity nucleotides that confer specificity but also by a series of minor elements that contribute to the efficient expression of this specificity. In the case of *E. coli* $tRNA^{Ala}$, mutating the major identity element G3·U70 drastically reduces alanylation, and conversely, introduction of the G3·U70 base pair into a new tRNA sequence transforms this tRNA into an alanine acceptor. However, optimal alanylation requires the presence of A73, G1·C72, G2·C71, G20, and G60 (Shi et al., 1990; McClain et al., 1991; Tamura et al., 1991a; Francklyn et al., 1992b), which thus can be considered as minor elements. The chimeric transcript Asp^{AF}_1 already contains some of these sequence features, namely, A73, G1·C72, and G20.

Exchange of base pair C2·G71 of transcript Asp^{AF}_1 to G2·C71 in transcript Asp^{AF}_2 contributed largely to the enhancement of alanylation. The improvement was about 2 orders of magnitude better than that arising from structural rearrangements of the tRNA (Figure 7).

The unexpected result was the concomitant strong improvement of phenylalanylation activity of mutant Asp^{AF}_2 (66-fold), because base pair 2·71 was not considered to participate in phenylalanine identity (Sampson et al., 1989). The beneficial effect brought by base pair G2·C71 on phenylalanylation of the chimeric $tRNA^{Asp}$ may result from structural consequences on the accessibility of chemical groups in the acceptor stem toward the synthetase. Another possibility would be that base pair C2·G71 in the $tRNA^{Asp}$ framework is an antideterminant for yeast PheRS; this is, however, unlikely because yeast PheRS can efficiently charge transplanted tRNAs possessing this feature (Sampson et al., 1989). A mutational analysis of the acceptor stem of this tRNA and of $tRNA^{Phe}$ is underway to explore these different possibilities and to find the exact molecular reasons for this effect (M. Frugier, R. Giegé, and C. Florentz, manuscript in preparation).

In conclusion, this set of experiments confirms the importance of the sequence context for alanine identity within a novel host, namely, $tRNA^{Asp}$. It shows in addition that sequence features in the amino acid accepting arm are essential for phenylalanine identity. However, the sole sequence change of base pair 2·71 is not sufficient to generate a $tRNA^{Asp}$ which is still not optimally recognized and charged by AlaRS and PheRS. Rather than trying to optimize the expression of both specificities by further sequence changes whose purpose would be to eliminate potential negative determinants, we

created variants presenting both the sequence change at base pair 2·71 and the structural changes in the D-loop and/or the variable loop.

Structure, Context, and other Considerations. Combining mutations at both the structural level (D-loop and variable loop) and at the sequence for base pair 2·70 allowed us to convert the chimeric transcript $Asp^{AF}{}_1$ into an optimized substrate for both *E. coli* AlaRS and yeast PheRS (Figure 7). Although small differences remain when comparing individually k_{cat} and K_M values of the mutants $Asp^{AF}{}_4$ and $Asp^{AF}{}_6$ with those of the wild-type transcripts, the relative k_{cat}/K_M values become equivalent (Table I). These differences may fall within the level of experimental errors but more likely reflect subtle mechanistic differences in the charging processes.

Interestingly, both structural and sequence effects seem to be cumulative for alanine and phenylalanine activities. This implies that the product of the *L*-values of structural and sequence mutants should correspond to the *L*-values for the wild-type $Asp^{AF}{}_1$ transcript [for theoretical explanations, see Pütz et al. (1993)]. Indeed, within the range of experimental error on *L*-values, this is roughly the case. For instance, for alanylation activities (see Table I), $L(Asp^{AF}{}_3) \times L(Asp^{AF}{}_2) = 1280 \times 7 = 8960$, which corresponds well to $L(Asp^{AF}{}_1) = 8850$. This conclusion is corroborated by the *L*-values of the multiple mutants combining structural and sequence modifications that are equivalent to the wild-type values.

Very efficient valylation of the engineered $tRNA^{Asp}$ was not obtained. This may simply reflect that ValRS does not share exactly the same requirements as PheRS and AlaRS. It shows also that it may be difficult to achieve aminoacylation that is highly efficient for all three amino acids because sequence changes that are helpful to one specificity may be harmful to another. Indeed, G20, G34, A35, and/or G3·U70 may be negative signals toward ValRS.

$Asp^{AF}{}_4$, already optimally charged with PheRS and AlaRS, and rather well charged with ValRS, might be a substrate for other synthetases. A search for the presence of major identity nucleotides responsible for additional specificities points to the presence of several nucleotides responsible for tyrosylation by *E. coli* TyrRS, namely, A73, G1·C72, G2 (Celis et al., 1973), and G34 (Sherman et al., 1992b). However, the structural framework of *E. coli* $tRNA^{Tyr}$ diverges largely from that of either $tRNA^{Asp}$ or the engineered $Asp^{AF}{}_4$, due to conformational differences at the level of the D-loop and to the presence of a long variable domain. Further, the series of Asp^{AF} variants contains other partial sets of identity nucleotides. This is the case of anticodon residue A35 and discriminator base A73 involved in the methionine identity set in both *E. coli* (Schulman & Pelka, 1988) and yeast (Senger et al., 1992) and of residues G34 and A36 included respectively in the identity sets of tryptophan (Himeno et al., 1991; Pak et al., 1992) and threonine (Schulman & Pelka, 1990; Hasegawa et al., 1992) in *E. coli*. However, the lack of other major identity residues for these identities, conformational differences, and the interplay of determinants and antideterminants for different specific aminoacylations most likely will prevent the Asp^{AF} variants from being efficiently charged by these other amino acids. Additional experiments are required to verify explicitly these predictions.

The success of the experiments reported in the present work in achieving multiple aminoacylation specificities is a reflection of the dispersion in the full tRNA of the identity nucleotides. This is in contrast to the situation occurring in RNA microhelices based solely on acceptor stem sequences, where attempts to achieve aminoacylation of microhelices with more than one amino acid were not successful. With such small structures, the nucleotide determinants are overlapping and mutually exclusive (Franklyn et al., 1992b).

Besides the yeast aspartate, phenylalanine, and valine systems, our study included the alanine system from *E. coli*. The experiments conducted with a crude enzyme preparation from yeast led to conclusions similar to those from experiments involving AlaRS from *E. coli*. Thus, identity elements for alanylation, first discovered in *E. coli*, are conserved in yeast, as suggested by Trézéguet et al. (1991). This is also in agreement with former conclusions pointing to the universality of the major alanine identity elements (Hou & Schimmel, 1989a).

Manipulation of identities should lead to the creation of other rationally designed species of multipartite tRNAs (which might be created alternatively by *in vitro* selection methods). From a practical point of view, tRNAs with multiple specificities could represent useful tools for engineering protein synthesis. The fact that the multiple mutant designed in this work is active in the presence of a crude enzymatic system encourages its use as a degenerate amino acid donor in *in vitro* protein synthesis. This could be the starting point for establishing *in vitro* selection methods for degenerate proteins statistically modified at given amino acid positions.

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