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# The RNA sequence context defines the mechanistic routes by which yeast arginyl-tRNA synthetase charges tRNA

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## ABSTRACT

**Arginylation of tRNA transcripts by yeast arginyl-tRNA synthetase can be triggered by two alternate recognition sets in anticodon loops: C35 and U36 or G36 in $tRNA^{Arg}$ and C36 and G37 in $tRNA^{Asp}$ (Sissler M, Giegé R, Florentz C, 1996, *EMBO J 15*:5069–5076). Kinetic studies on tRNA variants were done to explore the mechanisms by which these sets are expressed. Although the synthetase interacts in a similar manner with $tRNA^{Arg}$ and $tRNA^{Asp}$, the details of the interaction patterns are idiosyncratic, especially in anticodon loops (Sissler M, Eriani G, Martin F, Giegé R, Florentz C, 1997, *Nucleic Acids Res 25*:4899–4906). Exchange of individual recognition elements between arginine and aspartate tRNA frameworks strongly blocks arginylation of the mutated tRNAs, whereas full exchange of the recognition sets leads to efficient arginine acceptance of the transplanted tRNAs. Unpredictably, the similar catalytic efficiencies of native and transplanted tRNAs originate from different $k_{cat}$ and $K_m$ combinations. A closer analysis reveals that efficient arginylation results from strong anticooperative effects between individual recognition elements. Nonrecognition nucleotides as well as the tRNA architecture are additional factors that tune efficiency. Altogether, arginyl-tRNA synthetase is able to utilize different context-dependent mechanistic routes to be activated. This confers biological advantages to the arginine aminoacylation system and sheds light on its evolutionary relationship with the aspartate system.**

**Keywords: aminoacylation identity; evolution; RNA–protein recognition; $tRNA^{Arg}$; $tRNA^{Asp}$**

## INTRODUCTION

Accuracy of translation relies on specific aminoacylation of tRNAs by their cognate aminoacyl-tRNA synthetases (aaRS). This specificity is governed by molecular signals within tRNAs, including positive elements responsible for specific recognition by cognate synthetases and negative elements hindering recognition by noncognate enzymes. Recognition sets have been established in a number of tRNAs (e.g., Giegé et al., 1993; McClain, 1993a, 1993b; Saks et al., 1994) and were shown to be constituted by a limited number of nucleotides and/or structural features. The concept of specificity calls for a unique combination of those elements for a given aminoacylation system and indeed many experimental data are in line with this view. However, for the arginine system, we have demonstrated that yeast arginyl-tRNA synthetase (ArgRS) has the unexpected property of recognizing indiscriminately two alternate sets of nucleotides within two host tRNAs (Sissler et al., 1996). Indeed, ArgRS aminoacylates its major $tRNA^{Arg}$ isoacceptor thanks to the presence in its anticodon loop of C35 and, to a lesser extent, of U36 or G36. This set is designated as $[C35U36]^{Arg}$. Additionally, yeast ArgRS is also able to interact rather strongly with native $tRNA^{Asp}$ and to mischarge this molecule with low efficiency (Ebel et al., 1973; Gangloff et al., 1973; Perret et al., 1990a). Arginylation becomes efficient with noncognate $tRNA^{Asp}$ so far as it is deprived of modified nucleotides (Perret et al., 1990a; Pütz et al., 1994). For this unmodified substrate, arginylation is deeply related to the presence of residues C36 and G37, but is insensitive to the nature of nt 35. This second and alternate recognition set is designated as $[C36G37]^{Asp}$. Furthermore, contacts of yeast ArgRS on in vitro-transcribed $tRNA^{Arg}$ (derived from the major isoacceptor) and $tRNA^{Asp}$ have been established by footprinting with enzymatic and chemical probes (Sissler et al., 1997). They revealed that both transcripts interact with ArgRS along the D-arm side as typical for class I synthetases, and the anticodon loop, the region that contains the identity nucleotides. However, details

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of interaction patterns are idiosyncratic and indicate that recognition is governed by the synthetase.

The existence of two alternate identity sets that trigger catalysis by the same synthetase addresses new questions about the mechanisms leading to aminoacylation specificity. We recall that transplantation of individual arginine identity elements from one tRNA substrate into the other one has negative consequences on arginylation (Sissler et al., 1996) and suggests nonclassical behaviors of these elements. Further, the functional properties of a chimeric $tRNA^{Arg}$ with the anticodon loop of $tRNA^{Asp}$ demonstrated that the framework in which the identity elements are embedded contributes to expression of arginine identity (Sissler et al., 1996).

This work investigates the mechanism of arginine identity expression and the precise interrelations between the two arginine identity sets within the $tRNA^{Arg}$ or $tRNA^{Asp}$ frameworks. Functional analysis of a series of single and multiple $tRNA^{Arg}$ and $tRNA^{Asp}$ variants were aimed at defining the relationships between the individual identity elements in the two tRNA frameworks. Data indicate that arginylation of the in vitro transcripts is governed by alternate mechanisms according to the host tRNA. Not only does ArgRS interact differently with tRNA anticodon loops, but it also triggers efficient aminoacylation by alternate mechanisms as reflected by the kinetic analysis of the arginylation reactions. These findings open new evolutionary perspectives toward understanding tRNA aminoacylation identity.

## RESULTS AND DISCUSSION

### Four efficient tRNA substrates of ArgRS behave differently kinetically

Exchange of individual arginine identity nucleotides between $tRNA^{Arg}$ and $tRNA^{Asp}$ transcripts produces dramatic negative effects on their arginylation capacities (Sissler et al., 1996). To understand the duality of arginine identity expression, two chimera were constructed into which the complete recognition set of the other host molecule was transplanted. In these tRNAs, residual nucleotides from the native recognition set were removed by mutation. Sequences of variants,[1] together with those of their wild-type counterparts, are displayed in Figure 1. Variant **Arg***U35C36G37*, constituted by the $tRNA^{Arg}$ framework, contains in its anticodon loop the $[C36G37]^{Asp}$-recognition set. In this molecule, position 35 has been mutated to *U* in order to remove **C35**, the major identity nucleotide of the $[C35U36]^{Arg}$-recognition set. Inversely, variant *Asp***C35U36A37**, constituted by the $tRNA^{Asp}$ framework, bears the $[C35U36]^{Arg}$-recognition set and has *G37* replaced by **A** in order to remove the residual identity nucleotide of the $[C36G37]^{Asp}$-recognition set.

The kinetic characteristics of the two chimera and the wild-type tRNAs are given in Table 1. The losses ($\mathcal{L}$) of arginylation efficiencies (or kinetic specificities) are expressed in comparison to wild-type in vitro-transcribed $tRNA^{Arg}$, considered as the reference molecule throughout this work. All four tRNAs are efficient substrates of yeast ArgRS with charging efficiencies varying at most by a factor of 11. Thus, transplantation of the complete recognition sets is well perceived by ArgRS, even if transplantations of single nucleotides dramatically impair arginylation of host tRNAs (e.g., $\mathcal{L} = 2780$ after insertion of **U36** into $tRNA^{Asp}$, see Table 1). However, analysis of the individual kinetic parameters of the four tRNAs shows great variability.

A deeper insight comes from comparison of arginylation efficiencies within couples of variants. Comparing **Arg***U35C36G37* ($\mathcal{L} = 11$) and *AspWT* ($\mathcal{L} = 10$) with **ArgWT** (grey arrows in Fig. 1) shows that the moderate loss in activity for the $tRNA^{Arg}$ variant is due to strong compensations between affinity and catalytic rate ($1/K_m$ increases 10.5-fold; $k_{cat}$ decreases 115-fold), whereas with *AspWT* affinity remains constant and the 10-fold loss is only due to a decreased $k_{cat}$. Similarly, comparing *Asp***C35U36A37** with *AspWT* and **Arg***U35C36G37* (dashed arrows) reveals different levels of compensation brought by $K_m$ and $k_{cat}$. Altogether, different combinations of relative $k_{cat}$ and $K_m$ lead to similar losses of catalytic efficiencies.

Interestingly, **Arg***U35C36G37* with the $[C36G37]^{Asp}$-recognition set acquires the efficiency of *AspWT* ($\mathcal{L} =$ 11 versus 10), and *Asp***C35U36A37** bearing the $[C35U36]^{Arg}$-recognition set is as active as **ArgWT** ($\mathcal{L} = 0.8$ versus 1). In other words, the two couples of transcripts (linked by black arrows in Fig. 1), each sharing the same nucleotide triplet at positions 35, 36, and 37 (either **C35U36A37** or *U35C36G37*), have a similar catalytic efficiency ($\mathcal{L} \sim 10$ or $\mathcal{L} \sim 1$). This suggests that nucleotide combinations at positions 35–37 have a dominant role in arginylation whatever the tRNA framework.

Comparison of the kinetic data of the four arginine acceptors also informs about the role of the tRNA frameworks. First, **ArgWT** and *Asp***C35U36A37**, with the same arginine recognition set but not the framework, have a 4.7-fold difference in $K_m$ compensated by a 3.5-fold difference in $k_{cat}$. Larger effects are observed for *AspWT* and **Arg***U35C36G37*, where both $K_m$ and $k_{cat}$ vary by a factor of $\sim$11.

The existence of such differences between individual kinetic parameters of transplanted and wild-type tRNAs is surprising because the anticodon loops of the four tRNAs possess all the elements needed to preserve a

[1] Throughout the text, tRNA "body" means all nucleotides except those from the anticodon loop and "framework" means all nucleotides except the triplet 35, 36, and 37 containing the identity elements. Nomenclature of variants is as in Sissler et al. (1996), namely with bold characters for features occurring naturally in $tRNA^{Arg}$ and italics for nucleotides occurring naturally in $tRNA^{Asp}$; wild-type tRNA transcripts are referred to as **ArgWT** and *AspWT*.

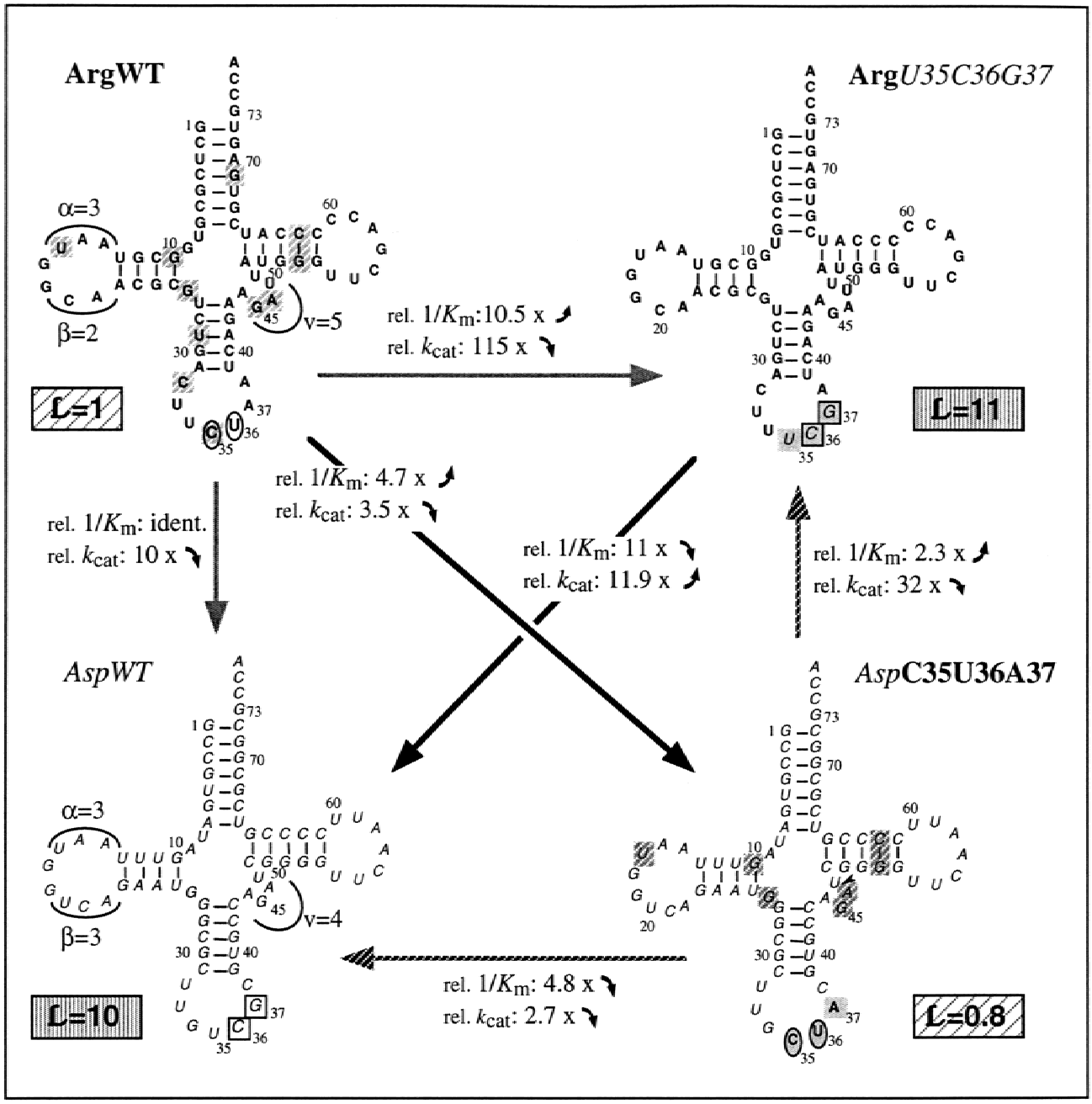


**FIGURE 1.** Relationship between four efficient tRNA substrates of yeast ArgRS. Wild-type in vitro transcripts of $tRNA^{Arg}$ (**ArgWT**) and $tRNA^{Asp}$ (*AspWT*) bearing each a different identity set within their anticodon loop (either circled or boxed) are the reference molecules. In both tRNAs, the original set has been removed and the alternate set introduced, allowing creation of variants **Arg***U35C36G37* and *Asp***C35U36A37** (with mutated positions shaded). Bold characters correspond to nucleotides occurring naturally in $tRNA^{Arg}$ and italics for those occurring naturally in $tRNA^{Asp}$. **L** values are losses in aminoacylation efficiencies with **ArgWT** as reference. The functional relationships between tRNAs are given as x-fold increases or decreases of rel. $1/K_m$ and rel. $k_{cat}$-values ("rel." stands for "relative") and are symbolized by arrows pointing up or down, respectively. Nucleotides common to the five yeast $tRNA^{Arg}$ isoacceptors (with the exception of conserved and semi-conserved residues) are shaded in the sequence of **ArgWT**; those common in $tRNA^{Arg}$ isoacceptors and $tRNA^{Asp}$ are shaded in the sequence of *Asp***C35U36A37** (arrow indicates that a nucleotide is missing in the variable region of $tRNA^{Asp}$). Sizes of $\alpha$ and $\beta$ domains and of variable region are indicated in **ArgWT** and *AspWT* (note that these sizes are not conserved in the arginine isoacceptors).

canonical anticodon loop structure (review by Dirheimer et al., 1995). Conserved U33 is maintained as well as semi-conserved pyrimidine 32 and purine 37. However, residues 32 and 38, which form a noncanonical base pair stacked over the anticodon stem, are different (C32–A38 or U32–C38) and, for some of the couples, the tRNA framework is different. We hypothesize that these differences account for the observed kinetic

**TABLE 1**. Kinetic parameters for arginylation by yeast ArgRS of Arg/Asp chimera and of architectural tRNA variants derived from yeast $tRNA^{Arg}$ and $tRNA^{Asp}$.

| Transcripts | $k_{cat}$ ($s^{-1}$) | $K_m$ (nM) | $k_{cat}/K_m$ (relative) | $\mathcal{L}_{exp}$ (x-fold) | $\mathcal{L}_{calc}$ (x-fold) | $\mathcal{R}$ |
|---|---|---|---|---|---|---|
| $tRNA^{Arg}$ variants | | | | | | |
| **ArgWT**[a] | 0.68 | 550 | 1 | 1 | | |
| **Arg***U35*[a] | 0.0003 | 735 | 0.0005 | 2,670 | | |
| **Arg***C36*[a] | 0.014 | 520 | 0.022 | 45 | | |
| **Arg***G37*[a] | 0.30 | 605 | 0.4 | 2.5 | | |
| **Arg***U35C36G37* | 0.0059 | 52 | 0.092 | 11 | $30 \times 10^4$ | $3.6 \times 10^{-5}$ |
| "Hyb"[a] | 0.052 | 28,900 | 0.0015 | 670 | | |
| "Hyb"**C35** | 0.028 | 618 | 0.037 | 27 | | |
| "Hyb"**C35U36** | 0.45 | 1108 | 0.33 | 3 | | |
| $tRNA^{Asp}$ variants | | | | | | |
| *AspWT*[a] | 0.07 | 570 | 0.1 | 10 | | |
| *Asp***C35**[a] | 0.031 | 310 | 0.082 | 12 | | |
| *Asp***U36**[a] | 0.0006 | 1,415 | 0.0003 | 2,780 | | |
| *Asp***A37**[a] | 0.0004 | 2,560 | 0.0001 | 7,230 | | |
| *Asp***U36A37** | 0.0004 | 324 | 0.001 | 990 | $20 \times 10^6$ | $4.9 \times 10^{-5}$ |
| *Asp***C35U36A37** | 0.19 | 118 | 1.30 | 0.8 | $241 \times 10^6$ | $1.24 \times 10^{-9}$ |
| Architectural variants | | | | | | |
| **Arg***20/48* | 4.39 | 2,038 | 1.7 | 0.6 | | |
| "Hyb"*20/48* | 0.018 | 3,047 | 0.0047 | 210 | | |

[a]Data from Sissler et al. (1996). Relative kinetic specificity constants are defined as $(k_{cat}/K_m)_{relative} = (k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{wild\text{-}type}$. $\mathcal{L}$ values are inverses of $(k_{cat}/K_m)_{relative}$. $\mathcal{R}$ values are defined as $\mathcal{R} = \mathcal{L}_{exp}/\mathcal{L}_{calc}$. Values of $k_{cat}$ and $K_m$ were determined from Lineweaver and Burk plots and varied for replicate experiments at most 10%. For all sets of kinetic analyses with tRNA variants, a control with **ArgWT** was run in parallel. All displayed data are mean values of at least two independent experiments and are normalized with regard to **ArgWT**. For nomenclature of variants, see footnote.

variabilities, a view supported by the crystallographic structure of the glutamine complex, representative of complexes with class I synthetases, in which nt 32 and 38 of $tRNA^{Gln}$ are base paired and stacked over the anticodon stem (Rould et al., 1989, 1991).

In summary, the above results indicate that arginylation by yeast ArgRS can be governed by multiple effects combining information transduction from recognition sets in the anticodon loop to the catalytic site of the synthetase with differential participation of the tRNA bodies. There are at least four productive combinations of tRNA sequences that are aminoacylatable by ArgRS. In what follows, we examine in detail the contribution of identity elements and evaluate more precisely the role of the tRNA structures.

## Additive relationship between arginine recognition sets and tRNA frameworks

An interesting relationship between **ArgWT** and *AspWT* appears when considering the tRNA as a structure built by two blocks, namely the framework (F) (as defined previously) and the N35–37 nucleotide triplet (T) containing the identity elements. Thus, **ArgWT** can be symbolized by ArgF/ArgT and *AspWT* by AspF/AspT, and one tRNA appears derived from the other by two large mutations (Fig. 2). With the formalism $\mathcal{L}_{calc}(mm) = \mathcal{R}\Pi\mathcal{L}_{exp}(sm)$, where $\mathcal{L}$'s are losses of catalytic efficiency of multiple (mm) and single (sm) mutants and $\mathcal{R}$ is a coupling factor accounting for deviations from additivity (Fersht, 1985; Pütz et al., 1993), one can calculate the impairment of arginylation for *AspWT* compared with **ArgWT** in two different ways. First, it can be calculated as the product of the loss of the triple $tRNA^{Arg}$ mutant **Arg***U35C36G37* ($\mathcal{L}$ = 11) by that of *AspWT* with respect to this triple mutant ($\mathcal{L}$ = 10/11 = 0.9).

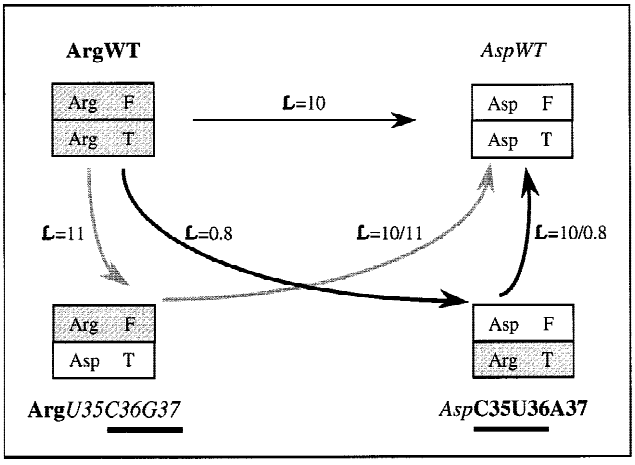


**FIGURE 2.** Additivity between structural blocks in tRNA substrates of yeast ArgRS. The two structural blocks constituting tRNA are symbolized by rectangles with F for frameworks and T for the N35–37 triplets. In the names of mutants with exchanged blocks, the recognition nucleotides are underlined. $\mathcal{L}$ values are taken from Table 1 and Figure 1.

Second, it can be calculated using the alternate combination, with the loss of the "tRNA$^{Asp}$ framework" mutant *Asp***C35U36A37** ($\mathcal{L}$ = 0.8) multiplied by that of *AspWT* with respect to **Arg***U35C36G37* ($\mathcal{L}$ = 10 × 0.8 = 12.5). In both cases, the calculated $\mathcal{L}$'s [$\mathcal{L}_{calc}$ = 11 × 0.9 = 9.9 or $\mathcal{L}_{calc}$ = 0.8 × 12.5 = 10] are equivalent to the experimental $\mathcal{L}$ = 10, which implies $\mathcal{R} \sim 1$ or additivity. In other words, arginine-accepting tRNAs are constituted by two structural blocks that act independently. Two combinations of blocks can lead to this additivity, namely the tRNA$^{Arg}$ framework and the triplet from tRNA$^{Asp}$, and conversely, the tRNA$^{Asp}$ framework and the triplet from tRNA$^{Arg}$.

### Anticooperative relationship between anticodon loop nucleotides

To understand the contribution of individual anticodon loop nucleotides to arginylation, an analysis of partially transplanted tRNAs has been performed. Results are given in Table 1 and Figure 3. This analysis, again, is based on comparison of calculated versus experimental aminoacylation efficiencies of multiple mutants according to $\mathcal{L}_{calc}(mm) = \mathcal{R}\Pi\mathcal{L}_{exp}(sm)$, with $\mathcal{R} \sim 1$ for additivity, $\mathcal{R} > 1$ for cooperativity, and $\mathcal{R} < 1$ for anticooperativity (Pütz et al., 1993).

Conversion of **ArgWT** to **Arg***U35C36G37* was done progressively (Fig. 3). Individual substitutions of **C35** and **U36** reduce arginylation, especially in **Arg***U35* ($\mathcal{L}$ = 2670) and more moderately in **Arg***C36* ($\mathcal{L}$ = 45). These mutations were useful to define **C35** as the major arginine recognition element and **U36** as a minor element (Sissler et al., 1996). Replacement of **A37** by *G37* is without noticeable effect ($\mathcal{L}$ = 2.5 in **Arg***G37*). Simultaneous mutation of the three anticodon loop positions leads to efficient **Arg***U35C36G37* ($\mathcal{L}$ = 11). Comparison of its experimental efficiency with that calculated from single mutant data (Table 1) highlights an anticooperative relationship between the three nucleotides. Indeed, in the hypothesis of additivity, $\mathcal{L}_{calc}$ would be 2.5 × 45 × 2,670 = 3 × 10$^5$, a figure much higher than $\mathcal{L}_{exp}$ = 11. The coupling factor $\mathcal{R}$ of 3.6 × 10$^{-5}$ quantifies this strong effect. A simple interpretation would be that the anticooperativity is due to compensating effects brought by the mutations in the [C35U36]$^{Arg}$-recognition set. A more subtle interpretation has to take into account that the mutations have replaced this set by the alternate [C36G37]$^{Asp}$-set. Because footprinting shows proximity of identity nucleotides with ArgRS (Sissler et al., 1997), it is reasonable to assume that anticooperativity is due to replacement of the [C35U36]-dependent pattern of contacts with ArgRS by the alternate [C36G37]-dependent pattern.

The same type of transplantation was done in the tRNA$^{Asp}$ context (Fig. 3). Four mutants were studied. Two of them, *Asp***U36** and *Asp***A37**, are simple mutants. They have strongly impaired arginylation ($\mathcal{L}$ = 2,780 and 7,230, respectively), although the mutations have introduced in the aspartate framework residues naturally present in tRNA$^{Arg}$. The two other variants, *Asp***U36A37** and *Asp***C35U36A37**, are double and triple mutants. In *Asp***U36A37**, the initial [C36G37]$^{Asp}$-recognition set has been removed. As anticipated, this double variant is a poor substrate for ArgRS. However, impairment of its activity ($\mathcal{L}$ = 990) is much lower than expected and anticooperativity occurs. Comparison of $\mathcal{L}_{calc}$ (2,780 × 7,230 = 20 × 10$^6$) with $\mathcal{L}_{exp}$ (=990) quantifies the anticooperative relationship between **U36** and **A37** by a coupling factor $\mathcal{R}$ = 4.9 × 10$^{-5}$. This strong anticooperativity can only result from compensatory effects brought by the mutations at the recognition positions because no positive signal has been introduced, except **U36** considered as a minor identity element in the tRNA$^{Arg}$ context. Replacement in tRNA$^{Asp}$ of *U35* by **C35** does not affect arginylation ($\mathcal{L}$ = 12 compared with $\mathcal{L}$ = 10 for *AspWT*). Because of the neutral effect of the **C35** mutation, it could be anticipated that its introduction in the double mutant *Asp***U36A37** would not improve its arginylation capacity. However, the resulting triple variant, *Asp***C35U36A37**, is a very efficient substrate ($\mathcal{L}$ = 0.8). The calculated coupling factor $\mathcal{R}$ = 1.24 × 10$^{-9}$ reveals here also strong anticooperative effects. Because *Asp***C35U36A37** is bearing the complete [C35U36]$^{Arg}$-recognition set, it is reasonable to assume that anticooperativity is mediated by the creation of novel productive interactions with ArgRS.

In summary, transformation of wild-type tRNA$^{Arg}$ or tRNA$^{Asp}$ transcripts into active chimera with exchanged arginine recognition sets results from strong anticooperative effects between nt 35, 36, and 37 that likely result from different adaptabilities of the tRNAs on ArgRS.

### Importance of nonrecognition residues on expression of arginine identity

For a better perception of arginine identity, hybrid tRNAs combining the body of tRNA$^{Arg}$ with wild-type or mutated versions of tRNA$^{Asp}$-derived anticodon loops were studied. The results are presented in Table 1 and Figure 4. The reference molecule is **Arg***U35C36G37*, which is a good arginine acceptor compared with **ArgWT** ($\mathcal{L}$ = 11, see above). Notice that the anticodon loop of this molecule is an Arg/Asp chimera with four residues from tRNA$^{Arg}$ and three from tRNA$^{Asp}$, namely dinucleotide *C36G37*, the alternate arginine recognition set [C36G37]$^{Asp}$, and *U35*, which is a non-arginine nucleotide in the tRNA$^{Arg}$ framework.

Activity of **Arg***U35C36G37* was first compared with that of three hybrid tRNAs (Fig. 4). The variant named "Hyb" contains the body of tRNA$^{Arg}$ and the wild-type aspartate anticodon loop. The two other hybrids ("Hyb"**C35** and "Hyb"**C35U36** ) have, respectively, one and two residues from the [C35U36]$^{Arg}$-recognition

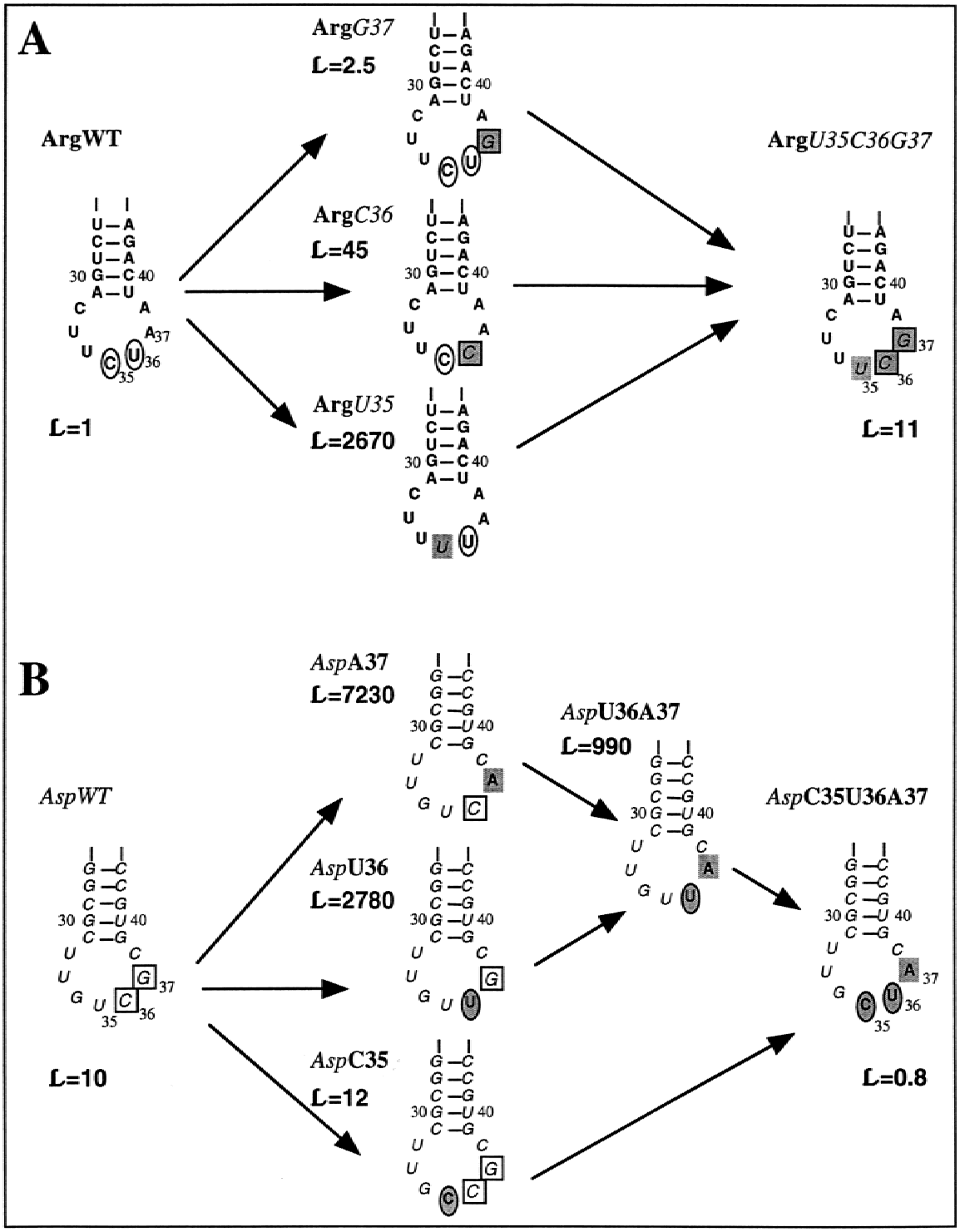


**FIGURE 3.** Anticooperativity between anticodon loop nucleotides in tRNA arginylation reactions catalyzed by yeast ArgRS. **A:** From $tRNA^{Arg}$-recognition set $[C35U36]^{Arg}$ in **ArgWT** to $tRNA^{Arg}$-recognition set $[C36G37]^{Asp}$ in **Arg***U35C36G37* via single mutants **Arg***U35*, **Arg***C36*, and **Arg***G37*. **B:** From $tRNA^{Asp}$-recognition set $[C36G37]^{Asp}$ in *AspWT* to $tRNA^{Asp}$-recognition set $[C35U36]^{Arg}$ in *Asp***C35U36A37** via single mutants *Asp***C35**, *Asp***U36**, and *Asp***A37** and the double mutant *Asp***U36A37.** Graphic symbols are as in Figure 1.

set. As to "Hyb," its sole difference with **Arg***U35C36G37* concerns the three residues 32, 34, and 38, U33 being common to all tRNAs. Because "Hyb" contains the same arginine determinants as **Arg***U35C36G37* and because a neutral behavior of the changes in its anticodon loop (see Fig. 4) could be expected, a significant arginine activity for this variant was anticipated. On the contrary, "Hyb" shows a strong decrease in aminoacylation effi-

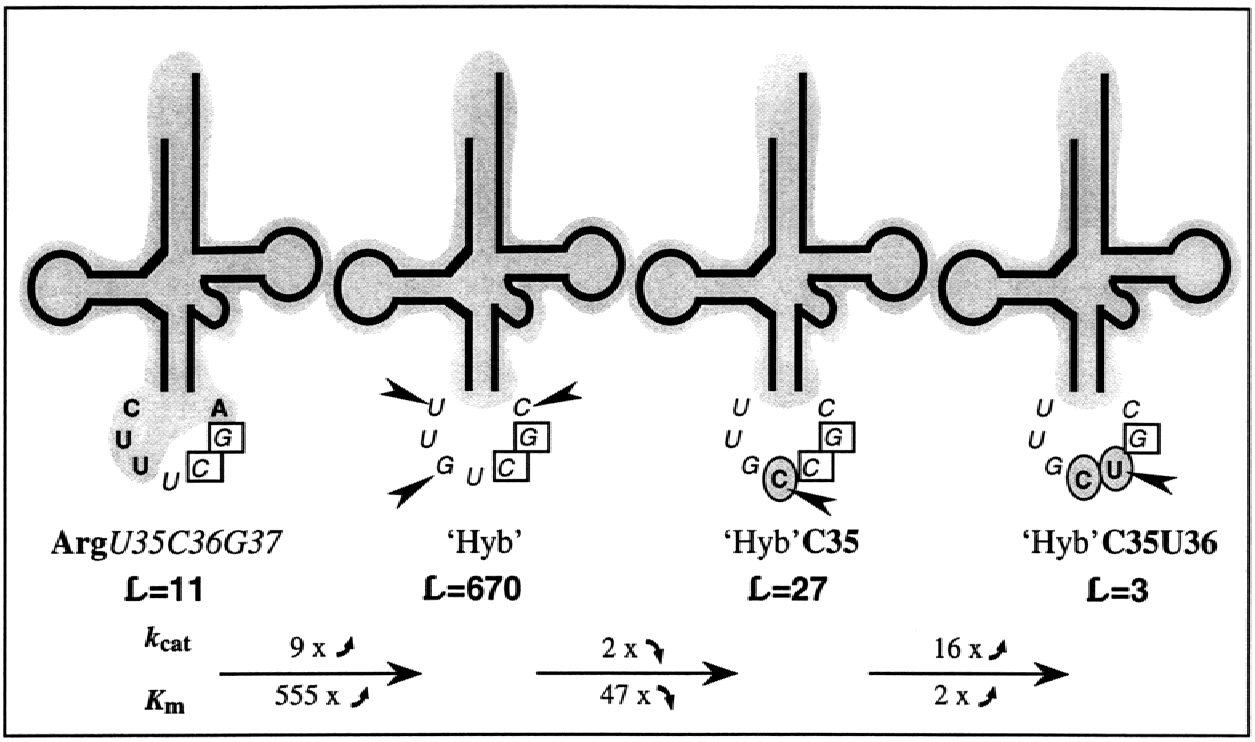


**FIGURE 4.** Effect of anticodon residues outside arginine recognition sets on aminoacylation by yeast ArgRS. The four tRNAs have the body of $tRNA^{Arg}$ (depicted as the backbone) with anticodon elements derived from either $tRNA^{Arg}$ or $tRNA^{Asp}$. Sequence elements derived from $tRNA^{Arg}$ are on a grey background. Other typographic characters are as in Figure 1. The changes (x-fold) of kinetic parameters between two variants (molecules at the left compared with their neighbors at the right) are indicated; arrows (up or down) symbolize that values of $k_{cat}$ or $K_m$ are increased or decreased, respectively.

ciency ($\mathcal{L} = 670$) accounted for by a 13-fold decrease in $k_{cat}$ and a 52-fold increase in $K_m$. The correlated large loss in affinity, suggested by the increased $K_m$, reflects either the presence of strong negative elements and/or a drastic conformational change in the anticodon loop of this tRNA that hinders recognition by ArgRS. Whatever the exact reason for the poor activity of "Hyb," it is related to the tRNA sequence outside the *U35C36G37* triplet that triggers activation of ArgRS. The same conclusion highlighting a major communication between anticodon loops and tRNA bodies arises when comparing activity of *AspWT* to that of "Hyb." Indeed, despite the fact that both tRNAs have the same anticodon loop, *AspWT* is 67-fold better arginylated than "Hyb" (compare $\mathcal{L}$ values given in Table 1).

The strong negative effect brought by the body of $tRNA^{Arg}$ can be overcome in part by the single mutation U35 → C35 ("Hyb"**C35**, Fig. 4). In this variant, arginylation efficiency is 25-fold improved ($\mathcal{L} = 27$). This improvement, however, does not completely recover the activity ($\mathcal{L} = 11$) of the reference molecule. Insertion of **C35** does not affect $k_{cat}$ significantly, but decreases $K_m \sim 50$-fold. It is worth recalling that, in the $tRNA^{Asp}$ context, replacement of *U35* by **C35** has no effect. Complete arginine activity is recovered by inserting **U36** in "Hyb"**C35** ($\mathcal{L} = 3$). In this fully active molecule, the real $[C35U36]^{Arg}$-recognition set reappears.

Altogether, study of tRNA hybrids indicates a strong functional link between the body of $tRNA^{Arg}$ and the anticodon loop. It reveals also the crucial role of non-recognition nucleotides in this loop for optimal expression of the $[C36G37]^{Asp}$-recognition set.

### Effect of the overall tRNA architecture on expression of arginine identity

The transcripts of $tRNA^{Arg}$III and $tRNA^{Asp}$ present structural differences in their D-loops and variable regions, with the $\alpha/\beta$ domains of D-loops (at both sides of conserved G18G19) formed, respectively, by three/two and three/three nucleotides and variable regions formed by five and four nucleotides (see Fig. 1). These regions participate in 3D-folding of tRNA (Giegé et al., 1993) and account for conformational differences between tRNAs (e.g., Romby et al., 1985; Perret et al., 1992; Sissler et al., 1997) that were correlated with functional effects (e.g., Perret et al., 1992; Frugier et al., 1993, 1994). Along these lines, structural characteristics from $tRNA^{Asp}$ have been introduced into $tRNA^{Arg}$ derivatives and their effect on arginylation has been measured (Table 1). An additional U has been introduced between G19 and C20, and U48 has been removed from the variable region in both **ArgWT** (which becomes **Arg***20/48*) and "Hyb" (which becomes "Hyb"*20/48*).

Introduction of aspartate architectural features in **ArgWT** has rather moderate effects on arginylation, but closer analysis of the kinetic data reveals unpredicted features. Although an impairment of activity could be anticipated for **Arg***20/48*, experiments in contrast indicate a gain in efficiency ($\mathcal{L} = 0.6$ or G $\sim$ 2). For "Hyb"*20/48*, the effects are qualitatively similar, with a $\sim$threefold increase in activity compared with "Hyb" ($\mathcal{L} = 210$ versus $\mathcal{L} = 670$; Table 1). Interestingly, the better activities of the two architectural variants with reference to the control molecules (**ArgWT** and "Hyb") result from opposite variations of $k_{cat}$ and $K_m$ (Table 1). For **Arg***20/48*, both $k_{cat}$ and $K_m$ are increased $\sim$six- and $\sim$fourfold compared with **ArgWT**; for "Hyb"*20/48*, in contrast, they are decreased $\sim$3- and $\sim$10-fold compared with "Hyb." This indicates subtle influences of the tRNA framework on expression of arginine identity.

The unexpected outcome of these experiments is the preference of ArgRS for architectural features from $tRNA^{Asp}$, as evidenced by the better charging of **Arg***20/48* and "Hyb"*20/48* than that of the control molecules with the arginine framework. Noticeable is the much decreased $K_m$, thus the increased affinity of "Hyb"*20/48* for ArgRS in comparison with "Hyb," and, more surprising, the improvement of the catalytic turnover when **ArgWT** is mutated to **Arg***20/48*.

## GENERAL CONCLUSIONS AND PERSPECTIVES

Several conclusions highlighting mechanistic and evolutionary peculiarities of the arginine system emerge from the present study.

### Mechanistic aspects

The fact that various tRNA constructs, differing either in their identity element content, their fine structure, and/or their sequence context, are all efficient substrates for ArgRS demonstrates that arginylation can proceed through various mechanistic routes. The two recognition sets $[C35U36]^{Arg}$ and $[C36G37]^{Asp}$, which are in contact with ArgRS upon complex formation, are interchangeable within the two structural contexts of $tRNA^{Arg}$III and $tRNA^{Asp}$ provided they are transplanted as a whole. The arginylation kinetics of the four efficient ArgRS substrates, i.e., the two wild-type tRNAs and the two chimera with exchanged recognition sets, reveal that the tRNAs are combinations of structural blocks. These blocks are the frameworks and the N35–37 triplets that behave independently. Closer analysis of the kinetic properties shows that the mechanisms by which arginine identity is expressed are not the same. Indeed, slower catalytic turnovers are compensated by better affinities of the tRNAs to ArgRS, an effect particularly pronounced for **Arg***U35C36G37*. Conversely, faster turnovers are compensated by worse affinities. From a structural point of view, this means that recognition of the nucleotide combinations accounting for arginine identity is accompanied by adequate adaptation between tRNAs and ArgRS. This adaptation is based on conformational changes of the nucleic acid, as demonstrated explicitly for **ArgWT** (Sissler et al., 1997).

Functional adaptation implies creation of appropriate contacts between recognition elements on RNA and protein. Because both kinetics and recognition elements differ in these tRNAs, their contacts with ArgRS likely are not the same. Thus, signals they switch on are transduced from their anticodon regions to the catalytic site of the synthetase, $-70$ Å apart, by different routes. Architectural features of the tRNA cores modulate this sequence-dependent process, which relies on the deformability of the nucleic acid. This is a common feature in protein–RNA recognition (Draper, 1995). Although the precise sequence of events leading to functional specificity is not yet unraveled, it is likely that these events include transconformation steps of the tRNA structure and thus are of the "induced fit" type. Indeed, individual arginine recognition elements have strongly anticooperative effects and nonrecognition elements are of importance for the expression of arginine identity. Such residues participate in the overall adaptation of the tRNA on the synthetases. Thus, subtle sequence relationships exist in arginine-accepting tRNAs. Here we have identified a number of them and we cannot exclude that other sequence combinations would also sustain efficient arginylation. Combinatorial methods will be required to explore these possibilities. Interestingly, the in vivo arginylation of an amber suppressor $tRNA^{Phe}$ mutant lacking residue 26 was explained by a possible structural adjustment of the tRNA on the synthetase that allows simultaneous use of A20, the major arginine recognition element in *Escherichia coli*, and of an analogue of conserved C35, namely C34 from the CUA amber anticodon, which thus would contribute to arginine identity as well (McClain & Foss, 1988). In line with the present work, this former study shows that *E. coli* ArgRS, like the yeast enzyme, can accommodate alternative recognition sets.

The great tolerance of ArgRS for a large variety of substrates, as described in the present report, has likely been amplified because of the greater flexibility of tRNA transcripts deprived of posttranscriptional modifications, compared with fully modified tRNAs (review by Agris, 1996). Thus, it is possible that the mechanistic routes governing arginylation in vivo are more restraint. Not only structural stabilization, but also protection against noncognate aaRS by posttranscriptional modification, as is typically the case for $tRNA^{Asp}$ thanks to the presence of an antideterminant on position G37, could, in addition, influence acceptability of alternative identity nucleotide sets. Moreover, compensation of large $K_m$ effects by $k_{cat}$ effects may not be overcome in vivo.

However, the occurrence of five different $tRNA^{Arg}$ isoacceptors in yeast, diverging largely at sequence and structural levels (see below), opens the possibility that ArgRS recognizes and charges each of the isoacceptors through alternate mechanistic routes. This, in turn, as well as the data obtained for the in vitro transcripts, opens the possibility of several combinations of amino acid identity sets at the level of the synthetase.

### Evolutionary aspects

One can wonder why nature has established such intricate molecular recognition mechanisms for tRNA arginylation. In yeast, five isoaccepting $tRNA^{Arg}$ species with large divergence in their sequences co-exist. Functional plasticity in the arginylation system opens the possibility that arginylation of each isoacceptor tRNA occurs through alternate mechanistic routes. This functional plasticity confers a biological advantage because arginylation efficiency may thus be less sensitive to mutations within tRNA or synthetase.

From these considerations, it follows that the phenomena and concepts discussed for the arginine system in yeast are of more general significance. tRNA aminoacylation systems are not completely idiosyncratic and are interconnected. The fact that this study was done on unmodified $tRNA^{Arg}$ and $tRNA^{Asp}$ transcripts has facilitated discovery of such interconnections. Even though major recognition nucleotides for tRNA identity can be discovered rather easily, it is shown here that other nucleotides in tRNA are not of completely neutral nature. Similar phenomena were recently reported in phenylalanylation systems (Kholod et al., 1997; Frugier et al., 1998). With *E. coli* PheRS, the recognition modes of cognate $tRNA^{Phe}$ and phage T5 noncognate $tRNA^{Phe}$ are dependent upon the $Mg^{2+}$ level and thus are of different nature (Kholod et al., 1997). With yeast PheRS, tRNA charging is possible in different sequence contexts, provided the recognition set defined in cognate $tRNA^{Phe}$ is present together with permissive elements in the acceptor stem of the engineered tRNAs (Frugier et al., 1998). Along the same lines, it was found in the *E. coli* alanine system that removal of the major recognition element can be compensated by a distal second mutation (Hou & Schimmel, 1992). Further, synthetases can recognize structures far larger than canonical tRNA [e.g., tRNA-like structures in viruses (review by Florentz & Giegé, 1995)] or obtained in vitro by artificial evolution (e.g., Tinkle-Peterson et al., 1993). This potential has, in part, been erased by epigenetic phenomena (as in the yeast arginylation system by methylation of G37 in $tRNA^{Asp}$) and by co-evolution of tRNAs for their interactions with other partners of the translational machinery. Knowledge of this potential of "functional plasticity" in tRNA aminoacylation systems encourages search of artificial tRNA substrates of synthetases that could become cellular inhibitors because they are unable to interact with the overall translational machinery.

Finally, the properties of yeast ArgRS reflect the evolutionary history of the arginylation system, in particular its relationship with the aspartate system and class II AspRS. First, we note the large sequence divergence between $tRNA^{Arg}$ isoacceptors, as in yeast, that is conserved through evolution (see the sequence compilation by Sprinzl et al., 1998). Similarly, the importance of C35 for arginine identity was demonstrated in *E. coli* (Schulman & Pelka, 1989; Tamura et al., 1992) and a functional link with structural characteristics of the D-loop was established (McClain & Foss, 1988; Schulman & Pelka, 1989; Tamura et al., 1992; McClain, 1993b). However, alternate recognition sets were not discovered to date in this organism, likely because no experiments were done on $tRNA^{Arg}$ isoacceptors with G37, thus resembling $tRNA^{Asp}$ in the anticodon loop. Following these lines, it is appealing to note the preference of ArgRS for structural characteristics present in $tRNA^{Asp}$. Interestingly enough, $tRNA^{Arg}$IV, a minor isoacceptor recently found in the yeast genome (Goffeau et al., 1996; Hani & Feldmann, 1998), presents a high sequence homology with yeast $tRNA^{Asp}$ (56%), much higher than that of the other arginine isocceptors (~30%), with, in particular, G37 and the aspartate architectural characteristics in the D-loop and variable region ($\alpha = \beta = 3$; $v = 4$). This could mean that proto-$tRNA^{Arg}$ resembled $tRNA^{Asp}$, or, expressed differently, that primordial class I ArgRS captured as substrate a tRNA, specific for the more ancient class II AspRS. If so, $tRNA^{Arg}$IV would be closest to such a primitive tRNA. In this context, is also remarkable to note an aspartate/arginine relationship at the gene level, with $tRNA^{Arg}$III and $tRNA^{Asp}$ originating from a same dimeric tRNA precursor in yeast (Schmidt et al., 1980). Although some of these correlations may be fortuitous, they provide a new conceptual frame to experimentally explore links between class I and class II tRNA aminoacylation systems.

## MATERIALS AND METHODS

### Materials

Nucleotides, deoxy-, and dideoxynucleotides were from Boehringer-Mannheim. Rotiphorese Gel 40 solution of acrylamide and *N,N′*-methylene-bis-acrylamide (19:1) was from Carl Roth GmbH (Germany) and radioactive L-[$^3$H] arginine (57 Ci/mmol) was from Amersham. Yeast ArgRS was purified from an overproducing strain (Sissler et al., 1997) and had a specific activity of 4,000 U/mg. T7 RNA polymerase was purified from an overproducing strain supplied by Dr. F.W. Studier (Brookhaven) according to Becker et al. (1996).

### Cloning and in vitro transcription

tRNAs were obtained by in vitro transcription of synthetic genes. Genes encoding wild-type and variant species of yeast

$tRNA^{Arg}$III and $tRNA^{Asp}$, downstream from the T7 RNA polymerase promoter, were constructed and cloned according to Sissler et al. (1996). We note that $tRNA^{Arg}$III, the major among the five arginine isoacceptors, is represented by 11 of 19 $tRNA^{Arg}$ genes in the yeast genome (Goffeau et al., 1996; Hani & Feldmann, 1998; G. Keith & G. Dirheimer, in prep.). Sequences of tRNAs are according to Gangloff et al. (1971) and Keith and Dirheimer (1980). For $tRNA^{Asp}$, it has been shown that replacement of U1-A72 by G1-C72, more favorable for transcription, is without detrimental consequence on its activity (Perret et al., 1990a). Plasmids containing genes for tRNAs were in vitro transcribed after linearization as described in Perret et al. (1990b). Transcripts were purified on 12% polyacrylamide/urea gels to single nucleotide resolution, electroeluted, and ethanol precipitated. Concentration of tRNA stock solutions were determined by absorbance measurements at $A_{260nm}$.

## Aminoacylation reactions

Aminoacylation reactions of transcripts derived from $tRNA^{Arg}$ and $tRNA^{Asp}$ have been performed as described in Sissler et al. (1996). Basically, from 0.2 to 4 $\mu$M of transcripts were incubated in 50 mM Hepes-NaOH, pH 7.5, 30 mM KCl, 15 mM $MgCl_2$, 10 mM ATP, 2.5 mM glutathione, 50 $\mu$M $^3$H-labeled arginine, and 0.3–900 nM of yeast ArgRS. Notice that the amino acid concentration in aminoacylation assays is much higher than the $K_m$ of arginine, which is 1.5 $\mu$M (Gangloff et al., 1976), so that kinetic artefacts are prevented.

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