

# A yeast arginine specific tRNA is a remnant aspartate acceptor

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## ABSTRACT

**High specificity in aminoacylation of transfer RNAs (tRNAs) with the help of their cognate aminoacyl-tRNA synthetases (aaRSs) is a guarantee for accurate genetic translation. Structural and mechanistic peculiarities between the different tRNA/aaRS couples, suggest that aminoacylation systems are unrelated. However, occurrence of tRNA mischarging by non-cognate aaRSs reflects the relationship between such systems. In *Saccharomyces cerevisiae*, functional links between arginylation and aspartylation systems have been reported. In particular, it was found that an *in vitro* transcribed tRNA<sup>Asp</sup> is a very efficient substrate for ArgRS. In this study, the relationship of arginine and aspartate systems is further explored, based on the discovery of a fourth isoacceptor in the yeast genome, tRNA<sub>4</sub><sup>Arg</sup>. This tRNA has a sequence strikingly similar to that of tRNA<sup>Asp</sup> but distinct from those of the other three arginine isoacceptors. After transplantation of the full set of aspartate identity elements into the four arginine isoacceptors, tRNA<sub>4</sub><sup>Arg</sup> gains the highest aspartylation efficiency. Moreover, it is possible to convert tRNA<sub>4</sub><sup>Arg</sup> into an aspartate acceptor, as efficient as tRNA<sup>Asp</sup>, by only two point mutations, C38 and G73, despite the absence of the major anticodon aspartate identity elements. Thus, cryptic aspartate identity elements are embedded within tRNA<sub>4</sub><sup>Arg</sup>. The latent aspartate acceptor capacity in a contemporary tRNA<sup>Arg</sup> leads to the proposal of an evolutionary link between tRNA<sub>4</sub><sup>Arg</sup> and tRNA<sup>Asp</sup> genes.**

## INTRODUCTION

Synthesis of functional proteins requires accurate translation of genetic information, a mechanism ensured not only by codon–anticodon interactions but also by high specificity in aminoacylation of transfer RNAs (tRNAs) with the help of their cognate aminoacyl-tRNA synthetases (aaRSs). This led to the concept of a second genetic code (1) and to attempts

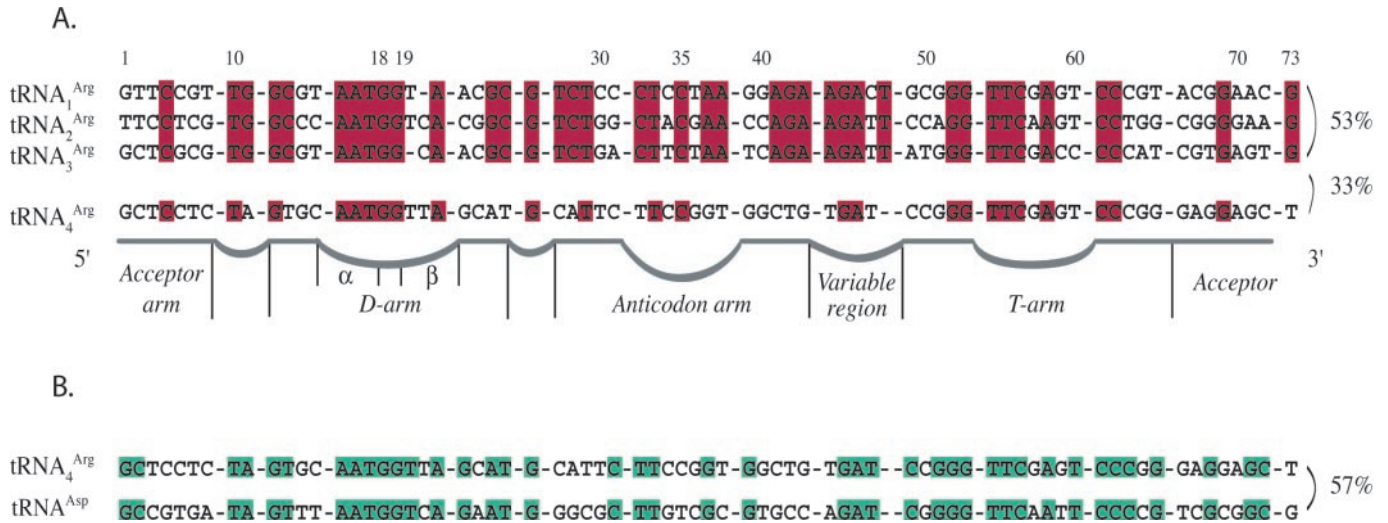
aimed to correlate the triplet genetic code with those specifying tRNA/aaRS interactions, in particular when it was discovered that tRNA identity determinants can be present within tRNA anticodons (2,3). By analogy with the triplet genetic code, it was anticipated that the tRNA identity rules underlying the second genetic code would be conserved in evolution, each tRNA aminoacylation system being idiosyncratic. Although the first expectation obtained strong experimental support (4); the second one is becoming obsolete, the accumulated evidences indicate the existence of functional and structural links between tRNA aminoacylation systems. For instance, identity of some tRNAs can be switched by a single mutation (3,5). Furthermore, the mischarging potential of many tRNAs by non-cognate aaRSs, demonstrated more than 30 years ago (6,7), can become a biological necessity in organisms lacking certain aaRS genes (8). Thus, when the gene for AsnRS is lacking, asparaginyl-tRNA<sup>Asn</sup> is formed in a process involving mischarging of tRNA<sup>Asn</sup> by AspRS followed by transamidation of the tRNA-bound aspartate into asparagine (9). The same holds true for the organisms deprived of a gene for GlnRS, where glutamate is mischarged on tRNA<sup>Gln</sup> by GluRS before being transamidated into glutamine (10). Similar situations exist for selenocysteine incorporation into proteins, with serine first being charged to tRNA<sup>Sec</sup> by a SerRS and then only transformed into selenocysteine (11). On the aaRS level, structure-based classification of these enzymes into groups and subgroups (12,13) also highlights the existence of evolutionary links (14–16). Altogether, these facts suggest the existence of evolutionary links between tRNA aminoacylation systems and in particular between tRNAs.

To approach the question of functional and evolutionary links between tRNAs, we focused our attention on the yeast *Saccharomyces cerevisiae* arginylation and aspartylation systems, the functional relationships between them have already been established. Despite the apparent unrelatedness of aspartate (17,18) and arginine (19) major identity elements, cross-aminoacylations between the two systems have been noted. Best studied is the arginylation of tRNA<sup>Asp</sup> by ArgRS. The low but significant mischarging of yeast tRNA<sup>Asp</sup> by ArgRS discovered in the 1970s (7,20) can be dramatically improved when the tRNA is unmodified. Indeed, the tRNA<sup>Asp</sup> transcript can be charged by ArgRS as efficiently as the tRNA<sub>3</sub><sup>Arg</sup> isoacceptor (the four yeast tRNA<sup>Arg</sup> isoacceptor species with

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**Figure 1.** Sequence comparison and tertiary structural elements of yeast aspartate and arginine isoacceptor tRNAs. (A) tRNA<sup>Arg</sup> isoacceptors 1, 2 and 3 (the three major species decoding 96.1% of the arginine codons) show 53% identity. Isoacceptor 4 is distinguished from the other three in sharing only 33% of common nucleotides (this minor species decodes only 3.9% of the arginine codons). Percentage of identical residues was calculated considering all nucleotides (also those conserved in all canonical tRNAs) shared between the compared sequences. The secondary structural domains are indicated below the primary sequences. (B) Sequence comparison of tRNA<sub>4</sub><sup>Arg</sup> and the single tRNA<sup>Asp</sup> isoacceptor indicates 57% identity. These two sequences share a same length in their variable region and in the  $\alpha$  and  $\beta$  domains of the D-loop.

anticodons CCU, ACG, UCU and CCG are termed as tRNA<sub>1</sub><sup>Arg</sup>, tRNA<sub>2</sub><sup>Arg</sup>, tRNA<sub>3</sub><sup>Arg</sup> and tRNA<sub>4</sub><sup>Arg</sup>, respectively), and therefore can be considered as an additional arginine acceptor (21). The protection of native tRNA<sup>Asp</sup> against efficient recognition by ArgRS is due to the N<sup>1</sup> methyl group on G37 antideterminant activity (22). Notably, the misarginylation potential of tRNA<sup>Asp</sup> is not mediated by mimics of the classical arginine identity elements present in tRNA<sub>3</sub><sup>Arg</sup>, but by an alternative set of determinants (C36 and G37, instead of C35 and U/G36) (19).

The reverse situation, recognition of tRNA<sup>Arg</sup> by AspRS, has been less studied. Potential aspartylation properties of the four tRNA<sup>Arg</sup> isoacceptors were recently sought and none was found significantly aspartylated (23). Interestingly, however, idiosyncratic functional features appear when these isoacceptors are transplanted with the full aspartate identity set. Two isoacceptors become active in aspartylation, strengthening the functional relationship between the arginine and aspartate systems, but the other two remain inactive, revealing the presence of aspartate antideterminants in their sequence. In this study, we explore this arginine/aspartate relationship in yeast more thoroughly, taking advantage of the knowledge of the complete sequence of the yeast genome (24). This sequence confirmed the existence of 16 identical copies of a tRNA<sup>Asp</sup> gene and revealed the existence of a single-copy gene of a minor tRNA<sub>4</sub><sup>Arg</sup>, in addition to three already known tRNA<sup>Arg</sup> isoacceptors. This minor tRNA is vital for the cell (25). Sequence comparisons indicate ~53% identity among tRNA<sub>1</sub><sup>Arg</sup>, tRNA<sub>2</sub><sup>Arg</sup> and tRNA<sub>3</sub><sup>Arg</sup>, and reveal a divergent tRNA<sub>4</sub><sup>Arg</sup> sequence sharing only 33% of its residues with the three other isoacceptors (Figure 1A). Interestingly, however, tRNA<sub>4</sub><sup>Arg</sup> shares 57% of its residues with tRNA<sup>Asp</sup> (Figure 1B) and conversely tRNA<sup>Asp</sup> does not show significant sequence similarity to other arginine isoacceptors (46–49%). This structural relationship between tRNA<sub>4</sub><sup>Arg</sup> and tRNA<sup>Asp</sup> is also related to the size of the  $\alpha/\beta$  regions in their D-loops ( $\alpha = \beta = 3$ ) and that of their variable region ( $v = 4$ ), different in the three other tRNA<sup>Arg</sup> isoacceptors

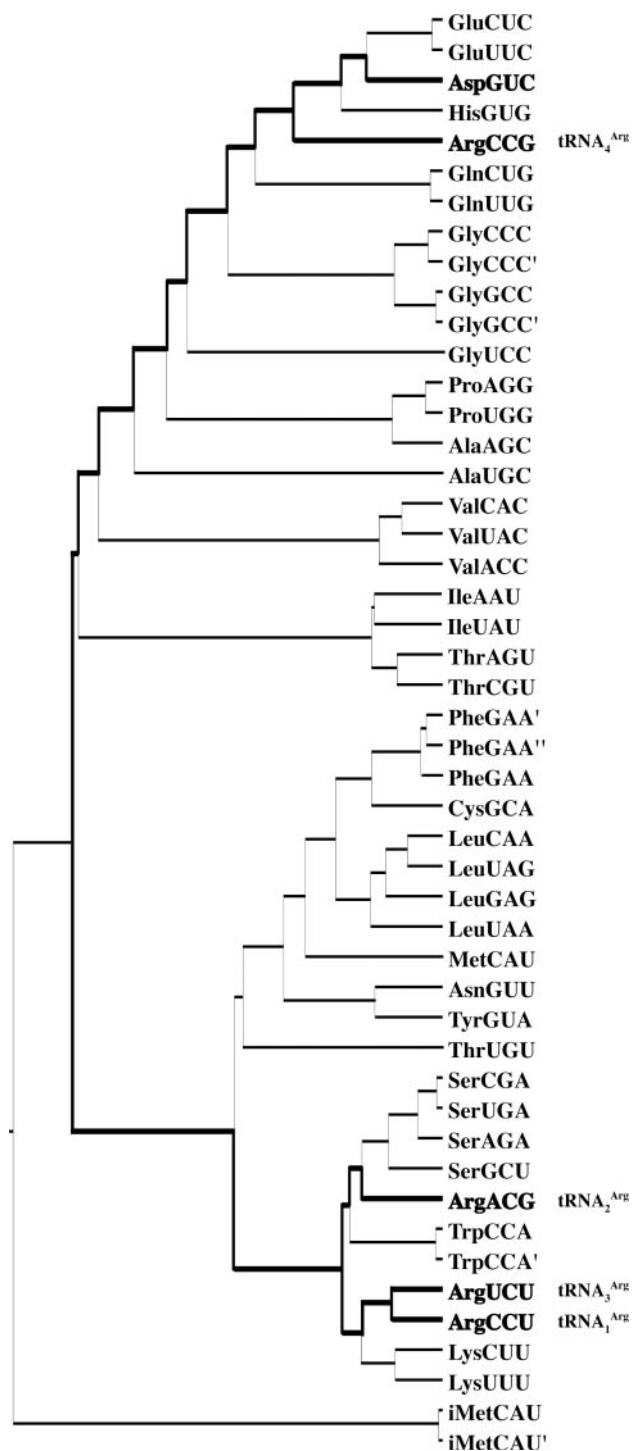
(Figure 1A and B). In summary, tRNA<sub>4</sub><sup>Arg</sup> sequence and structure more closely resemble those of tRNA<sup>Asp</sup> than those of the other three tRNA<sup>Arg</sup> species. This conclusion is strengthened by a dendrogram displaying the distances between the 48 yeast tRNA gene sequences (Figure 2). Although three arginine acceptor tRNA genes are clustered, the fourth sequence, corresponding to tRNA<sub>4</sub><sup>Arg</sup>, is located at a long distance, closer to the sequence of tRNA<sup>Asp</sup>. This suggests an evolutionary link between tRNA<sub>4</sub><sup>Arg</sup> and tRNA<sup>Asp</sup>.

The aim of the present study is to explore whether the sequence relationship between tRNA<sub>4</sub><sup>Arg</sup> and tRNA<sup>Asp</sup> correlates with functional properties, i.e. whether the yeast arginine isoacceptor bears a hidden aspartate-accepting properties. Only two point mutations are sufficient to convert transcribed tRNA<sub>4</sub><sup>Arg</sup> into a substrate for AspRS as efficient as homologous tRNA<sup>Asp</sup> showing that the two tRNAs are indeed functionally related. The functional relationship between the two tRNAs thus suggests an evolutionary linkage between the corresponding genes. This linkage is conserved in sequenced yeast species close to *S.cerevisiae* but is lost in the other fungi (distal species). Evolutionary implications are discussed.

## MATERIALS AND METHODS

### Materials

Synthetic genes of yeast tRNA<sup>Asp</sup> and tRNA<sub>3</sub><sup>Arg</sup> were cloned previously (19,26). Yeast AspRS was a gift from M. Frugier (IBMC, Strasbourg, France) (27). Yeast ArgRS was purified according to the method described previously (28). T7 RNA polymerase was purified from an overproducing strain as described previously (29). Oligonucleotides were synthesized and purified by Thermohybrid, Genset and Eurogentec. Restriction enzymes (BamHI, HindIII, BstNI and NsiI) and T4 polynucleotide kinase were from New England Biolabs. T4 DNA ligase was from Qbiogen. [L-<sup>3</sup>H]aspartic acid



**Figure 2.** Sequence distances in the 48 yeast tRNA genes highlight proximity between tRNA<sup>Asp</sup> and tRNA<sup>Arg</sup>. The dendrogram was established with the ClustalV program from MegAlign (Lasergene from DNASTar). Genes for tRNA<sup>Arg</sup> and tRNA<sup>Asp</sup> are highlighted as well as either short or long distance between them. A total of 48 tRNAs are identified by their anticodon triplet (the isoacceptor species with sequence variations outside the anticodon triplet are identified by single primes and double primes).

(37 Ci/mmol), [<sup>3</sup>H]arginine (41 Ci/mmol), [ $\gamma$ -<sup>32</sup>P]dATP and Hybond-XL nylon membrane were from Amersham. Yeast strain YAL5 n [*ura3-52 lys2-801<sup>am</sup> trp1- $\Delta$ 63 his3- $\Delta$ 200 leu2- $\Delta$ 1 ade2- $\Delta$ 450 ade3- $\Delta$ 1483, tRNA<sup>Arg</sup>::HIS3, pAL5*

(tRNA<sup>Arg+</sup>, Ura<sup>+</sup>, Ade3<sup>+</sup>) and plasmid pRS314-tRNA<sup>Arg</sup><sub>4</sub> (tRNA<sup>Arg+</sup>, Trp<sup>+</sup>) containing the wild-type tRNA<sup>Arg</sup><sub>4</sub> gene were obtained as described previously (25).

### Cloning and *in vitro* transcription

Experimental procedures for tRNA gene preparation were carried out as described previously (23). Clones containing tRNA genes were obtained by the hybridization of 10 overlapping phosphorylated oligonucleotides, ligation between BamHI and HindIII sites of plasmid pTFMa (26), and transformation into TG1 cells. Genes contain a T7 RNA polymerase promoter region followed by a hammerhead ribozyme sequence and the tRNA sequence. Transcription using T7 RNA polymerase was performed according to the standard procedures (26) for tRNA<sup>Arg</sup><sub>3</sub> and tRNA<sup>Asp</sup>, or with a higher concentration of MgCl<sub>2</sub> (30 mM versus 22 mM) for tRNAs cloned with an upstream ribozyme sequence (30). A BstNI site (or NsiI for tRNA<sup>Arg</sup><sub>2</sub> and for variant tRNA<sup>Arg/Asp</sup><sub>2</sub>) which possess an internal BstNI site coinciding with the 3' end of the tRNA allows synthesis of tRNAs ending with CCA. The hammerhead ribozyme self-cleaves the phosphodiester linkage directly upstream of nucleotide 1 and releases the tRNA (5'-OH). After phenol extraction, transcripts were purified on denaturing PAGE, electro-eluted and ethanol precipitated.

### Aminoacylation reactions

Aminoacylations were performed as described previously (23). Arginylation conditions were 50 mM HEPES-NaOH, pH 7.5, 30 mM KCl, 15 mM MgCl<sub>2</sub>, 10 mM ATP, 2.5 mM glutathione, 50  $\mu$ M [<sup>3</sup>H]arginine (74 GBq/mmol) and adequate amounts of transcript (50 nM to 6  $\mu$ M) and yeast ArgRS (2–730 nM). Aspartylation conditions were 50 mM HEPES-NaOH pH 7.5, 30 mM KCl, 15 mM MgCl<sub>2</sub>, 5 mM ATP, 32  $\mu$ M [<sup>3</sup>H]aspartic acid (208 GBq/mmol), and adequate amounts of transcript (20 nM to 6  $\mu$ M) and yeast AspRS (15 nM to 1  $\mu$ M). Aspartylation under mischarging conditions (7) was performed in the same medium except with a lower ATP concentration (0.5 mM versus 5 mM), in order to increase the [MgCl<sub>2</sub>]/[ATP] ratio. Transcripts were renatured by heating for 90 s at 60°C in water and slow cooling down to room temperature before performing aminoacylation at 30°C. Kinetic parameters,  $k_{cat}$  and  $K_M$ , were derived from Lineweaver-Burk plots. For the very poorly aminoacylated substrates, direct determination of aminoacylation efficiency,  $k_{cat}/K_M$  ratio was performed as described previously (3). Comparative charging levels were measured for 0.4  $\mu$ M tRNA in the presence of 200 nM enzyme for 20 min.

### *In vivo* experiments

*In vivo* experiments were performed as described previously (25). Plasmid pRS314-tRNA<sup>Arg</sup><sub>4</sub> was converted into pRS314-tRNA<sup>Arg/C38/G73</sup> containing mutant tRNA<sup>Arg/C38/G73</sup> gene using a QuikChange<sup>TM</sup> Site-Directed Mutagenesis kit from Stratagene. The *Escherichia coli* strain XL1-Blue was used as a recipient for cloning procedures. Bacteria were grown and transformed according to the standard procedures. An aliquot of 1  $\mu$ g of pRS314-tRNA<sup>Arg/C38/G73</sup> was used to transform the yeast red phenotype YAL5 strain, previously disrupted at tRNA<sup>Arg</sup><sub>4</sub> genomic sequence by the mutant gene  $\Delta$ tRNA<sup>Arg</sup><sub>4</sub>::HIS and rescued by the plasmid pAL5 (tRNA<sup>Arg+</sup>, Ura<sup>+</sup>,



Ade3<sup>+</sup>) (25), on minimal medium supplemented with adenine (limiting concentration of 2 µg ml<sup>-1</sup>), uracil (20 µg ml<sup>-1</sup>), lysine (30 µg ml<sup>-1</sup>) and leucine (60 µg ml<sup>-1</sup>) (selective medium). After 72 h incubation at 30°C, white sectors of colonies were isolated. White sectors correspond to cells cured of pAL5. These yeast cells contain only pRS314-*tRNA*<sub>4</sub><sup>Arg/C38/G73</sup> and so express only the double mutant *tRNA*<sub>4</sub><sup>Arg/C38/G73</sup>.

An aliquot of 250 ml of selective liquid medium were inoculated and grown at 30°C. The cells were collected either at stationary phase (OD<sub>700</sub> = 2) or during exponential phase (OD<sub>700</sub> = 0.7). Extraction of total RNA was performed on ice with acidic phenol (saturated with 0.3 M sodium acetate and 10 mM Na<sub>2</sub>EDTA, pH 5). These conditions allow stabilization of the ester linkage between the amino acid and the tRNA (31).

Northern-blot analyses were performed as described previously (25). Briefly, samples of 30 µg of total RNA were separated by PAGE (gel composition: 6.5% polyacrylamide, 8 M urea and 0.1 M sodium acetate, pH 5; migration buffer: 0.1 M sodium acetate, pH 5). After transfer on a nylon membrane, RNAs were detected with specific [5'-<sup>32</sup>P]oligo-labeled nucleotides. The probe for *tRNA*<sub>4</sub><sup>Arg</sup> was 5'-CTCGA-ACCCGGATCACAGCC, annealing to positions 39–59; the probe for 5S rRNA was 5'-ACCCACTACTACTCGGT-CAGGCTCTTAC; and for *tRNA*<sub>UAA</sub><sup>Leu</sup> was 5'-GGATGCCA-GTTTCGAACCTCGCGGG. The signals were quantified using a Fuji Bioimager Bas2000.

### In silico analyses

A dendrogram was prepared based on the sequences of all 48 tRNAs from *S.cerevisiae* obtained at <http://rna.wustl.edu/GtRDB> database. Sequences were imported into the EditSeq program (Lasergene from DNASTar) for converting them into a format appropriate for alignment using the MegAlign program (Lasergene from DNASTar). Sequences were aligned considering the canonical cloverleaf secondary structure elements. The dendrogram was performed using ClustalV (included in MegAlign).

Sequences of *tRNA*<sup>Arg</sup> (CCU, ACG, UCU, CCG, UCG, GCG) and *tRNA*<sup>Asp</sup> (GUC) genes were searched in several yeast genomes: *tRNA* gene sequences from *Schizosaccharomyces pombe* were found at <http://rna.wustl.edu/GtRDB>; those from *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Candida glabrata* and *Yarrowia lipolytica* were found in Génolevures database (<http://cbi.labri.fr/Genolevures/>); those from *Saccharomyces uvarum* and *Zygosaccharomyces rouxii* by blastn using *S.cerevisiae* sequences as query against nucleotide database (RST Génolevures) at <http://cbi.labri.u-bordeaux.fr/>.

For each organism, *tRNA*<sup>Asp</sup> was compared with each *tRNA*<sup>Arg</sup> isoacceptor. Percentages of identical residues were calculated in considering residues (including canonical nucleotides) shared between the two *tRNA* gene sequences. Programs used were EditSeq and MegAlign.

## RESULTS

### Aspartylation properties of *tRNA*<sup>Arg</sup> isoacceptor species

Aspartylation properties of the four *in vitro* transcribed *tRNA*<sup>Arg</sup> were tested under standard experimental conditions (5 mM ATP and 15 mM MgCl<sub>2</sub>) as well as under less stringent conditions (0.5 mM ATP and 15 mM MgCl<sub>2</sub>). Such conditions, favoring mischarging (7), are expected to reveal even weak aminoacylation properties. The results are given in Table 1. Under standard conditions, while the homologous transcript *tRNA*<sup>Asp</sup> is efficiently aspartylated (charging level up to 75%), the four yeast *tRNA*<sup>Arg</sup> species are very poor substrates for AspRS (charging levels <1%). However, measurement of the catalytic efficiency of aspartylation ( $k_{cat}/K_M$ ) reveals variations between the different *tRNA*<sup>Arg</sup> isoacceptors. Although *tRNA*<sub>2</sub><sup>Arg</sup> and *tRNA*<sub>3</sub><sup>Arg</sup> display a large decrease in catalytic efficiency ( $\geq 250\ 000$ -fold), *tRNA*<sub>1</sub><sup>Arg</sup> and *tRNA*<sub>4</sub><sup>Arg</sup> cause somewhat less drastic decrease (67 000-fold).

Under misaminoacylation conditions ([MgCl<sub>2</sub>]/[ATP] = 30), the four *tRNA*<sup>Arg</sup> become more efficiently misaspartylated. Most noticeable is the gain in aminoacylation efficiency of *tRNA*<sub>4</sub><sup>Arg</sup>. Although *tRNA*<sub>1</sub><sup>Arg</sup>, *tRNA*<sub>2</sub><sup>Arg</sup> and *tRNA*<sub>3</sub><sup>Arg</sup> remain charged to <1%, aspartylation of *tRNA*<sub>4</sub><sup>Arg</sup> reaches a level of 9%. When compared to *tRNA*<sup>Asp</sup>, losses in aminoacylation reach 20 000- to 100 000-fold for *tRNA*<sub>1</sub><sup>Arg</sup>, *tRNA*<sub>2</sub><sup>Arg</sup> or *tRNA*<sub>3</sub><sup>Arg</sup>, respectively. For *tRNA*<sub>4</sub><sup>Arg</sup> the loss is only 2200-fold. As an outcome, gains of efficiency (i.e. the efficiency of a tRNA substrate under permissive conditions compared to that under standard conditions) are variable: >2.5-fold for *tRNA*<sub>3</sub><sup>Arg</sup>, 3.3-fold for *tRNA*<sub>1</sub><sup>Arg</sup>, 12.5-fold for *tRNA*<sub>2</sub><sup>Arg</sup> and 30.5-fold for *tRNA*<sub>4</sub><sup>Arg</sup>. Owing to the weakest loss in efficiency under permissive conditions and the highest gain in activity when comparing efficiencies under standard and permissive conditions, *tRNA*<sub>4</sub><sup>Arg</sup> clearly distinguishes itself in aspartylation activity from the other *tRNA*<sup>Arg</sup> isoacceptors.

### *tRNA*<sub>4</sub><sup>Arg</sup> transplanted with the aspartate identity elements is distinct from the other arginine isoacceptors

The complete yeast aspartate identity set consists of residues G73, G10•U25, G34, U35, C36 and C38 (17,18). This identity

**Table 1.** Aspartylation of *in vitro* transcribed yeast *tRNA*<sup>Arg</sup> isoacceptors and yeast *tRNA*<sup>Asp</sup>

Transcript	Standard conditions <sup>a</sup> (5 mM ATP and 15 mM MgCl <sub>2</sub> )			'Permissive' conditions (0.5 mM ATP and 15 mM MgCl <sub>2</sub> )		
	Charging level (%)	$k_{cat}/K_M$ (10 <sup>-3</sup> s <sup>-1</sup> nM <sup>-1</sup> )	<i>L</i> (fold) <sup>b</sup>	Charging level (%)	$k_{cat}/K_M$ (10 <sup>-3</sup> s <sup>-1</sup> nM <sup>-1</sup> )	<i>L</i> (fold) <sup>b</sup>
<i>tRNA</i> <sup>Asp</sup>	75	0.5	1	100	1.0	1
<i>tRNA</i> <sub>1</sub> <sup>Arg</sup>	<1	7.5 × 10 <sup>-6c</sup>	67 000	<1	2.5 × 10 <sup>-5c</sup>	20 000
<i>tRNA</i> <sub>2</sub> <sup>Arg</sup>	<1	2.0 × 10 <sup>-6c</sup>	250 000	<1	2.5 × 10 <sup>-5c</sup>	20 000
<i>tRNA</i> <sub>3</sub> <sup>Arg</sup>	<1	nd	>250 000	<1	5.0 × 10 <sup>-6c</sup>	100 000
<i>tRNA</i> <sub>4</sub> <sup>Arg</sup>	<1	7.5 × 10 <sup>-6c</sup>	67 000	9	2.3 × 10 <sup>-4c</sup>	2200

nd, too low to detect.

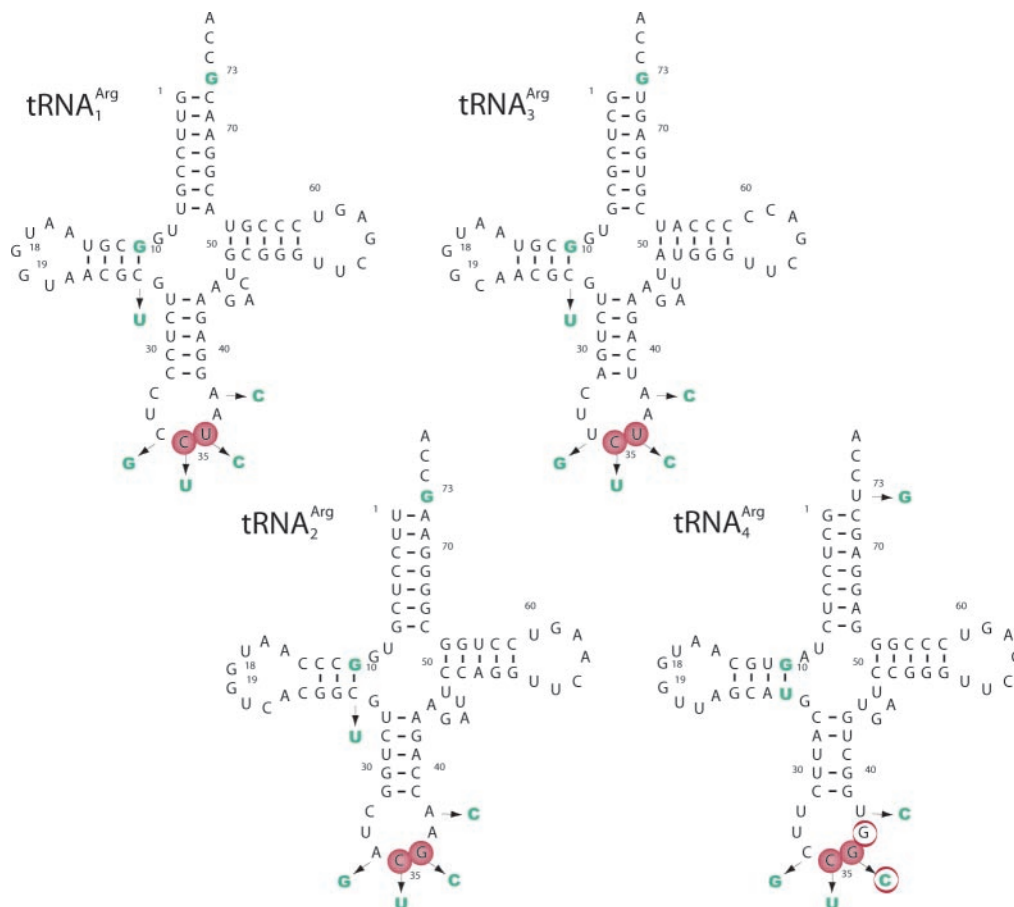
<sup>a</sup>Values from Fender *et al.* (23).

<sup>b</sup>*L* stands for the relative loss in aminoacylation efficiency considering *tRNA*<sup>Asp</sup> as the reference [for the definition of *L*, see (50)].

<sup>c</sup>Determined according to the method described in (3).

set was transplanted into each of the four tRNA<sup>Arg</sup> isoacceptors (Figure 3) and the residual arginylation capacities of the resulting chimeric molecules have been tested (Table 2). Their aspartylation properties have already been reported (23) and are recalled. tRNA<sub>1</sub><sup>Arg/Asp</sup>, tRNA<sub>2</sub><sup>Arg/Asp</sup> and tRNA<sub>3</sub><sup>Arg/Asp</sup>

almost completely lost their natural arginylation ability (charging levels <10%). Loss of catalytic efficiency compared to wild-type tRNA<sup>Asp</sup> varies from 7400-fold to more than 10 000-fold. On the other hand, tRNA<sub>4</sub><sup>Arg/Asp</sup> retains the ability to be efficiently arginylated with a charging level of 80% and a



**Figure 3.** Cloverleaf sequences of the four yeast tRNA<sup>Arg</sup> isoacceptors. Full red dots highlight the set of arginine identity elements experimentally determined for tRNA<sub>3</sub><sup>Arg</sup> (19). Insertion of the aspartate identity set (17,18) indicated in green, generates an alternative arginine identity set (19) in tRNA<sub>4</sub><sup>Arg</sup> (nucleotides in red circles).

**Table 2.** Arginylation and aspartylation of *in vitro* transcribed yeast tRNA<sup>Arg</sup> isoacceptors into which the whole set of aspartate identity elements was transplanted

Transcript	Charging level (%)	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_M$ (nM)	$k_{\text{cat}}/K_M$ (10 <sup>-3</sup> s <sup>-1</sup> nM <sup>-1</sup> )	$L$ (fold) <sup>a</sup>
<b>Arginylation</b>					
tRNA <sup>Asp</sup>	100	0.3	220	1.4	1
tRNA <sub>1</sub> <sup>Arg/Asp</sup>	<5	nd	nd	$1.4 \times 10^{-4b}$	10 000
tRNA <sub>2</sub> <sup>Arg/Asp</sup>	<1	nd	nd	nd	>10 000
tRNA <sub>3</sub> <sup>Arg/Asp</sup>	10	$8.9 \times 10^{-4}$	4680	$1.9 \times 10^{-4}$	7400
tRNA <sub>4</sub> <sup>Arg/Asp</sup>	80	0.2	190	1.1	1
<b>Aspartylation<sup>c</sup></b>					
tRNA <sup>Asp</sup>	75	0.03	60	0.5	1
tRNA <sub>1</sub> <sup>Arg/Asp</sup>	80	0.03	130	0.2	2.5
tRNA <sub>2</sub> <sup>Arg/Asp</sup>	<10	$9.2 \times 10^{-5}$	3225	$3.0 \times 10^{-5}$	16 700
tRNA <sub>3</sub> <sup>Arg/Asp</sup>	<10	$76 \times 10^{-5}$	1460	$52 \times 10^{-5}$	960
tRNA <sub>4</sub> <sup>Arg/Asp</sup>	80	0.06	130	0.5	1

nd, too low to detect. Aminoacylation reactions were performed under 'standard' conditions.

<sup>a</sup> $L$  stands for relative loss in aminoacylation efficiency considering tRNA<sup>Asp</sup> as the reference.

<sup>b</sup>Determined according to the method described in (3).

<sup>c</sup>Values from (23).

**Table 3.** Aspartylation and arginylation of *in vitro* transcribed yeast tRNA<sub>4</sub><sup>Arg</sup> and variants

Transcript	Charging level (%)	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_{\text{M}}$ (nM)	$k_{\text{cat}}/K_{\text{M}}$ (10 <sup>-3</sup> s <sup>-1</sup> nM <sup>-1</sup> )	$L$ (fold) <sup>a</sup>
Aspartylation					
tRNA <sup>Asp</sup>	75	0.03	60	0.5	1
tRNA <sub>4</sub> <sup>Arg</sup>	<1	nd	nd	$7.5 \times 10^{-6b}$	67 000
tRNA <sub>4</sub> <sup>Arg/C38</sup>	13	0.0003	290	0.001	500
tRNA <sub>4</sub> <sup>Arg/G73</sup>	72	0.004	300	0.01	50
tRNA <sub>4</sub> <sup>Arg/C38/G73</sup>	98	0.07	440	0.2	2.5
Arginylation					
tRNA <sub>4</sub> <sup>Arg</sup>	90	0.5	470	1.1	1
tRNA <sub>4</sub> <sup>Arg/C38</sup>	88	0.2	20	10.0	0.1
tRNA <sub>4</sub> <sup>Arg/G73</sup>	74	0.2	920	0.2	5.5
tRNA <sub>4</sub> <sup>Arg/C38/G73</sup>	65	0.3	190	1.6	0.7

nd, too low to detect. Aminoacylation reactions were performed under 'standard' conditions.

<sup>a</sup> $L$  stands for relative loss in aminoacylation efficiency taking tRNA<sup>Asp</sup> (upper part) or tRNA<sub>4</sub><sup>Arg</sup> (lower part) as the references.

<sup>b</sup>Determined according to the method described in (3).

catalytic efficiency comparable to that of tRNA<sup>Asp</sup> ( $L = 1$ ). Thus, in addition to being the best transplanted substrate for AspRS (23), tRNA<sub>4</sub><sup>Arg/Asp</sup> has unusual arginylation properties compared to the other arginine isoacceptors transplanted with aspartate identity elements.

### Two mutations are sufficient to convert tRNA<sub>4</sub><sup>Arg</sup> into an efficient aspartate acceptor

The possibility that tRNA<sub>4</sub><sup>Arg</sup> could be converted into an efficient aspartate acceptor by a small number of mutations was explored. Three variants of tRNA<sub>4</sub><sup>Arg</sup> have been prepared. Two mutants had substitutions U38→C38 (tRNA<sub>4</sub><sup>Arg/C38</sup>) or U73→G73 (tRNA<sub>4</sub><sup>Arg/G73</sup>). The double mutant tRNA<sub>4</sub><sup>Arg/C38/G73</sup> was also constructed (for details see Discussion).

Activities of single and double mutants were tested both in the presence of yeast AspRS and ArgRS (Table 3). With respect to aspartylation, all three variants become better substrates for AspRS than wild-type tRNA<sub>4</sub><sup>Arg</sup> with charging levels varying between 13 and 98%. Affinity of AspRS for each of the three variants estimated by  $K_{\text{M}}$  is very similar (290–440 nM). Strong differences in catalytic efficiency was observed with  $k_{\text{cat}}$  of  $0.3 \times 10^{-3}$  and  $4 \times 10^{-3}$  s<sup>-1</sup> for the single mutants tRNA<sub>4</sub><sup>Arg/C38</sup> and tRNA<sub>4</sub><sup>Arg/G73</sup>, respectively, and a large increase to  $70 \times 10^{-3}$  s<sup>-1</sup> for the double mutant tRNA<sub>4</sub><sup>Arg/C38/G73</sup>. Thus, each single mutation causes aspartylation capacity to increase significantly compared to wild-type tRNA<sub>4</sub><sup>Arg</sup>. Substitution U38→C38 leads to a 134-fold gain of catalytic efficiency and substitution U73→G73 to a 1340-fold gain. Combination of the two mutations leads to the best effect. The double mutant tRNA<sub>4</sub><sup>Arg/C38/G73</sup> becomes only 2.5-fold less efficiently aspartylated as tRNA<sup>Asp</sup> with a 26 800-fold gain compared to wild-type tRNA<sub>4</sub><sup>Arg</sup>.

The three mutants remained as efficient substrates for ArgRS, with only limited variations in catalytic efficiency compared to the reference molecule. Charging levels vary from 65 to 90%. The rate constants  $k_{\text{cat}}$  are similar to the four transcripts but Michaelis–Menten constants  $K_{\text{M}}$  are different. Although for variant tRNA<sub>4</sub><sup>Arg/G73</sup> and tRNA<sub>4</sub><sup>Arg/C38/G73</sup>  $K_{\text{M}}$  is with a factor 2 of that of tRNA<sub>4</sub><sup>Arg</sup>, this parameter is 23.5-fold lower than that for tRNA<sub>4</sub><sup>Arg/C38</sup>. As a consequence the single variant bearing a mutation in the anticodon loop is

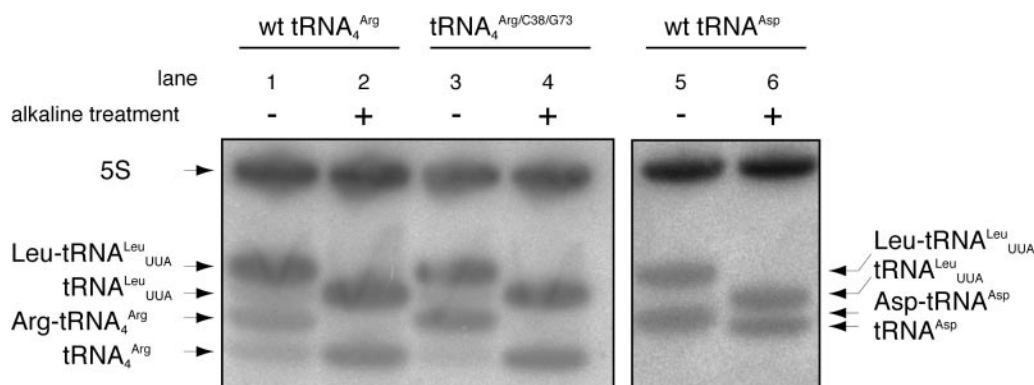
10-fold more efficient than wild-type, the mutant with the single mutation in the acceptor stem is 5.5-fold less efficient than wild-type and the double mutant is as active as wild-type.

In summary, it was possible to create a tRNA<sub>4</sub><sup>Arg</sup> variant in which only two positions have been modified, tRNA<sub>4</sub><sup>Arg/C38/G73</sup>, which remains a very efficient substrate for arginylation but which also becomes an aspartylation substrate as efficient as wild-type tRNA<sup>Asp</sup>.

### *In vivo*, the double mutated tRNA<sub>4</sub><sup>Arg/C38/G73</sup> is only arginylated

To investigate the effect of the dual arginine and aspartate acceptance of tRNA<sub>4</sub><sup>Arg/C38/G73</sup> on growth, we used a knockout strain deprived of the minor tRNA<sub>4</sub><sup>Arg</sup> gene of *S.cerevisiae* (25). Survival of this strain can be rescued by a copy of the gene present on a plasmid. Consequently, it is possible to analyze mutated tRNA<sub>4</sub><sup>Arg</sup> genes by plasmid shuffling to exchange the wild-type gene with the mutated gene. A successful exchange means that the tRNA<sub>4</sub><sup>Arg</sup> gene is active *in vivo* at each step from the initial transcription until the final incorporation of the amino acid into proteins. Here, we show that the rescuing plasmid pAL5 can be exchanged with the tRNA<sub>4</sub><sup>Arg/C38/G73</sup> gene carried in a pRS314 vector. The shuffle of pAL5 by pRS314-tRNA<sub>4</sub><sup>Arg/C38/G73</sup> was followed by a loss of red pigmentation (colonies appear white/red sector, then white) due to loss of *ADE3* (see Material and Methods). Therefore, tRNA<sub>4</sub><sup>Arg/C38/G73</sup> encoded by pRS314-tRNA<sub>4</sub><sup>Arg/C38/G73</sup> is capable of rescuing the tRNA<sub>4</sub><sup>Arg</sup>-knockout strain, which means that this tRNA is functional in translation *in vivo*.

tRNA extracted from the tRNA<sub>4</sub><sup>Arg</sup>-knockout strain containing exclusively tRNA<sub>4</sub><sup>Arg/C38/G73</sup>, was analyzed using northern blot under acidic conditions. Figure 4 compares the hybridization patterns of tRNA<sub>4</sub><sup>Arg</sup> and mutant tRNA<sub>4</sub><sup>Arg/C38/G73</sup> to tRNA<sup>Asp</sup> signals. The addition of an arginine residue to the tRNA molecule produces a consequent shift on the tRNA<sup>Arg</sup> migration (lanes 1 and 3), whereas that of an aspartate residue to tRNA<sup>Asp</sup> only produces less of a shift (lane 5). No signal corresponding to aspartyl-tRNA<sub>4</sub><sup>Arg/C38/G73</sup> is present in lane 3, indicating that significant aspartyl-tRNA<sub>4</sub><sup>Arg</sup> formation did not occur *in vivo*. However, quantification of the signals reveals that the arginyl-tRNA<sub>4</sub><sup>Arg</sup> levels were significantly changed



**Figure 4.** Northern-blot analysis of tRNA samples extracted from yeast cells under acidic conditions. Experimental procedure is described in Materials and Methods. Levels of aminoacylation of tRNA<sub>4</sub><sup>Arg</sup> and tRNA<sub>4</sub><sup>Arg/C38/G73</sup> were determined by measurement of the ratio of acylated (– lanes) and deacylated tRNA (+ lanes, obtained by alkaline treatment of the RNA extracts). The blot was probed with <sup>32</sup>P-labeled oligonucleotides complementary to 5S RNA (control of RNA content), to tRNA<sup>Leu</sup> (control of the acidic extraction procedure) and to tRNA<sub>4</sub><sup>Arg</sup>. Lanes 1 and 2, wild-type tRNA<sub>4</sub><sup>Arg</sup>; and lanes 3 and 4, mutated tRNA<sub>4</sub><sup>Arg/C38/G73</sup>. The second blot (lanes 5 and 6) was probed with a tRNA<sup>Asp</sup>-specific oligonucleotide. It shows that an aspartate residue attached to a tRNA molecule induces less of a shift than that of an arginine residue.

when comparing the native and mutant samples. Whereas the level of arginylation of wild-type tRNA<sub>4</sub><sup>Arg</sup> is usually ~70%, it becomes 85% for mutant tRNA<sub>4</sub><sup>Arg/C38/G73</sup> in the yeast cells (a reproducible observation for several independent experiments). An increased steady-state level in arginylation of tRNA<sub>4</sub><sup>Arg/C38/G73</sup> compared to the native one is observed which might be either due to more efficient acylation by ArgRS or due to lower sensitivity to deacylation.

## DISCUSSION

### Sequence similarity of tRNA<sub>4</sub><sup>Arg</sup> to tRNA<sup>Asp</sup> correlates with functional relationship

Sequencing the complete yeast genome revealed the existence of a fourth tRNA<sup>Arg</sup> isoacceptor, tRNA<sub>4</sub><sup>Arg</sup>. Sequence comparison and multiple sequence alignment emphasized the striking similarity of tRNA<sub>4</sub><sup>Arg</sup> to tRNA<sup>Asp</sup>. Thus, these two tRNAs were predicted to be evolutionarily related since they are clustered far from the three other tRNA<sup>Arg</sup> isoacceptors. Their resemblance is suggested not only by the number of identical residues but also by the similarity of structural motifs known to define tRNA tertiary structure elements, i.e. size of  $\alpha$  and  $\beta$  domains of the D-loop and size of the variable region (32). Analysis of the aminoacylation properties of a series of wild-type and mutant tRNA<sub>4</sub><sup>Arg</sup> and tRNA<sup>Asp</sup> *in vitro* transcripts demonstrates that tRNA<sub>4</sub><sup>Arg</sup> is also functionally distant from the three other arginine acceptors and close to tRNA<sup>Asp</sup>. Three types of results support this view: (i) aspartylation assays under different experimental conditions showed that while none of the arginine isoacceptors is aspartylated under classical conditions, tRNA<sub>4</sub><sup>Arg</sup> is the acceptor which becomes best aspartylated under permissive conditions (30.5 fold better) although it still remains 2200-fold less efficiently charged than tRNA<sup>Asp</sup>. (ii) When the full set of aspartate identity elements is transplanted into the tRNA<sup>Arg</sup> isoacceptors, only tRNA<sub>4</sub><sup>Arg</sup> gains full aspartate activity while keeping full arginylation capacity. Gain in aspartylation activity has been explained previously (23) not only by the insertion of aspartate identity elements but also by the absence of aspartate antideterminants in the sequence

background of tRNA<sub>4</sub><sup>Arg</sup>. Arginylation properties of the transplant would be expected to be lost due to the overlapping positions for aspartate and arginine identity elements in the anticodon loop. However, the transplanted tRNA<sub>4</sub><sup>Arg</sup> molecule expresses the alternate arginine identity set [C36G37 initially found within *in vitro* transcribed tRNA<sup>Asp</sup>, (19)] that results from the combination of residue G37, naturally present in tRNA<sub>4</sub><sup>Arg</sup> but absent within the three other tRNA<sup>Arg</sup>, with residue C36, introduced by transplantation. Absence of this alternate arginine identity set within the three other isoacceptors is in agreement with the non-arginylation properties of transplanted tRNA<sub>1</sub><sup>Arg</sup>, tRNA<sub>2</sub><sup>Arg</sup> and tRNA<sub>3</sub><sup>Arg</sup>. (iii) It was possible to convert tRNA<sub>4</sub><sup>Arg</sup> with a minimal number of mutations into an efficient aspartate accepting tRNA.

Despite its significant resemblance to tRNA<sup>Asp</sup>, wild-type tRNA<sub>4</sub><sup>Arg</sup> is a very poor substrate for AspRS. However, only a small number of mutations were expected to effect the conversion predicted by the closeness of the two tRNAs within the dendrogram. Residues of tRNA<sub>4</sub><sup>Arg</sup> to be mutated have to be peculiar to this tRNA, i.e. absent from tRNA<sup>Asp</sup> and from the three other tRNA<sup>Arg</sup>. Two nucleotides, U73 and U38, were found to be good candidates (Figure 5). Position 73 is crucial for tRNA<sup>Asp</sup> recognition by AspRS, G73 being an aspartate identity element (17) in direct contact with AspRS (33), and sufficient to mediate aspartylation of a tRNA minihelix (34). Thus, G73 was introduced into tRNA<sub>4</sub><sup>Arg</sup>. Notably, G73 present in tRNA<sub>1</sub><sup>Arg</sup>, tRNA<sub>2</sub><sup>Arg</sup> and tRNA<sub>3</sub><sup>Arg</sup>, is however not sufficient to allow for their aspartylation. Along the same lines, position 38 was chosen as site for mutation since it is peculiar to tRNA<sub>4</sub><sup>Arg</sup>. The three other arginine isoacceptor tRNAs display the doublet A37A38 within the anticodon loop, while tRNA<sub>4</sub><sup>Arg</sup> possesses the sequence G37U38. The residue G37 is found in tRNA<sup>Asp</sup> but followed by C38, which contributes to a low extent to aspartate identity [5-fold effect; (18)]. Upon substitution, these residues are predicted to enable aspartylation of tRNA<sub>4</sub><sup>Arg</sup>. Both substitutions were beneficial for tRNA<sub>4</sub><sup>Arg</sup> aspartylation. Replacement of either U73 or U38 in tRNA<sub>4</sub><sup>Arg</sup> by G73 or C38 leads to significant increase in catalytic efficiency (1340- and 134-fold, respectively). Combination of these two mutations converts



	tRNA <sub>1</sub> <sup>Arg</sup>	tRNA <sub>2</sub> <sup>Arg</sup>	tRNA <sub>3</sub> <sup>Arg</sup>	tRNA <sub>4</sub> <sup>Arg</sup>	tRNA <sup>Asp</sup>
N <sub>73</sub>	G	G	G	U → <sup>G</sup> <sub>G=1340</sub>	G → <sup>U</sup> <sub>L=36</sub>
Anticodon loop	C A U A C C U	C A U A A C G	C A U A U C U	U U → <sup>C</sup> <sub>G=134</sub> U G C C G	U C → <sup>U</sup> <sub>L=5</sub> U G G U C
N <sub>10</sub> -N <sub>25</sub>	G-C	G-C	G-C	G•U	G•U
aspartate acceptance	-	-	-	+/-	+++

**Figure 5.** Yeast aspartate identity elements in the four tRNA<sup>Arg</sup> isoacceptors. Nucleotides at position 73 (discriminator base), the anticodon loop and 10–25 bp are indicated. For comparison, the sequence of the single tRNA<sup>Asp</sup> isoacceptor is also indicated. Aspartate identity elements are highlighted with gray squares. Note the absence of aspartate determinants from the anticodon loop of each of the four tRNA<sup>Arg</sup> isoacceptors. tRNA<sub>4</sub><sup>Arg</sup> shares two nucleotides with tRNA<sup>Asp</sup> (open squares) in addition to U33 conserved in all tRNAs. Aspartate acceptance of each wild-type tRNA is indicated (+/-). Gains (G) or losses (L) in aspartylation capacity observed after single nucleotide mutations of tRNA<sub>4</sub><sup>Arg</sup> and tRNA<sup>Asp</sup> are indicated.

tRNA<sub>4</sub><sup>Arg</sup> into a substrate for AspRS almost as efficient as tRNA<sup>Asp</sup> (tRNA<sub>4</sub><sup>Arg/C38/G73</sup> is only 2.5-fold less efficiently aspartylated than tRNA<sup>Asp</sup>).

Notably, in addition to gaining aspartylation specificity, the double mutant tRNA<sub>4</sub><sup>Arg/C38/G73</sup> retains full efficiency of arginylation. These two mutations (U38→C38 and U73→G73) do not alter the arginine identity set C35G36 [previously established within tRNA<sub>3</sub><sup>Arg</sup>; (19)] and do not introduce antideterminants that would hinder recognition by ArgRS. Replacement of U73 by G73 has a small negative effect on arginylation properties ( $L = 5.5$ -fold) whereas introduction of C38 increases tRNA<sub>4</sub><sup>Arg</sup> catalytic efficiency ( $G = 10$ -fold). The two effects are due to variations in the affinity for ArgRS. They tend to cancel each other leading to a substrate as efficient as wild-type tRNA<sub>4</sub><sup>Arg</sup>.

#### Cryptic aspartate identity elements embedded in tRNA<sub>4</sub><sup>Arg</sup>

tRNA<sub>4</sub><sup>Arg</sup> is, among the four arginine isoacceptors, the only one capable to be aspartylated under conditions favoring misaminoacylation, an unexpected result when the aspartate identity elements were considered. Indeed, while tRNA<sub>1</sub><sup>Arg</sup>, tRNA<sub>2</sub><sup>Arg</sup> and tRNA<sub>3</sub><sup>Arg</sup> possess the major G73 aspartate identity element, tRNA<sub>4</sub><sup>Arg</sup> displays only the minor G10•U25 element (17). This was a first argument illuminating the possibility that cryptic aspartate elements are embedded in tRNA<sub>4</sub><sup>Arg</sup>. Proof came from the double mutant tRNA<sub>4</sub><sup>Arg/C38/G73</sup>, for which the gain in aspartylation is expected to result from the contribution of two individual new nucleotides (or the removal of either two neutral elements or two antideterminants) combined with additional other positive elements already present within tRNA<sub>4</sub><sup>Arg</sup>. Contribution to aspartylation efficiency of residues G73 and C38 have been estimated previously by studies performed on tRNA<sup>Asp</sup>. Replacement of these residues led to the loss of catalytic efficiency of 36-fold for G73 [when replaced by U73; (17)] and 5-fold for C38 [when replaced by U38; (18)]. These low losses in aspartylation efficiency do not fully explain the

gain of 1340- and 134-fold observed when inserting the corresponding residues into tRNA<sub>4</sub><sup>Arg</sup>. Strikingly, the double variant tRNA<sub>4</sub><sup>Arg/C38/G73</sup> is efficiently aspartylated despite the absence of the strongest aspartate identity elements, the anticodon triplet G34U35C36 residues, the mutation of which leads to the loss of up to 530-fold catalytic efficiency (17,35). These nucleotides are in direct contact with the AspRS (36,37). Altogether, these data strongly support the hypothesis that efficient aspartylation of tRNA<sub>4</sub><sup>Arg/C38/G73</sup> results from an alternative set of identity elements from that established within the tRNA<sup>Asp</sup> framework. These cryptic elements still have to be deciphered.

#### Sequence and functional similarities of tRNA<sub>4</sub><sup>Arg</sup> and tRNA<sup>Asp</sup> reveal an evolutionary relationship

We have uncovered remnant aspartate acceptor properties within a contemporary arginine-specific tRNA and propose an evolutionary linkage between tRNA<sub>4</sub><sup>Arg</sup> and tRNA<sup>Asp</sup> genes. This is argued by the sequence similarity between the two tRNA genes, the evolutionary proximity of the two tRNAs as shown by the dendrogram in Figure 2 and the reduced number of mutations required to bring to light the latent aspartate potential of tRNA<sub>4</sub><sup>Arg</sup>. Indeed, U73 and U38 are believed to play a major role during the evolutionary establishment of the restricted arginine specificity of tRNA<sub>4</sub><sup>Arg</sup>, and to have been introduced during evolution to hide the peculiar aspartylation potential of tRNA<sub>4</sub><sup>Arg</sup>. Mutation of these nucleotides allowed us to artificially turn back evolutionary, so that tRNA<sub>4</sub><sup>Arg/C38/G73</sup> would appear to be an intermediate in the history of tRNA<sub>4</sub><sup>Arg</sup>. This intermediate molecule would stand at the crossroad, capable of expressing both aspartate and arginine specificities.

Diversity of tRNA isoaccepting species would be expected to appear concomitantly with the expansion of the genetic code (38). Specific tRNAs would originate from a pool of precursors with relaxed amino acid acceptance. At an early stage of evolution, this relaxed discrimination would have been a driving force for molecular evolutionary processes, for instance in allowing the emergence of novel proteins



from the libraries of variant proteins (39). Setting up the 20 aminoacylation specificities of today depends in part on creation and enrichment of the population of tRNAs. Diversity of tRNAs may have occurred from gene duplications of tRNA precursors with ambiguous specificities (38,40–42). Duplication of a given tRNA gene might generate either a tRNA with a new specificity (occurrence of a new aminoacylation system), an additional isoacceptor tRNA (enrichment of the isoaccepting family), or an alloacceptor tRNA captured by a different pre-existing system. The outcome would be an enriched pool of highly specific tRNAs in which each codon would be decoded by a unique tRNA (38).

Along these lines, a tRNA sequence in yeast may once have been both an arginine and an aspartate acceptor. Ambiguity of this tRNA molecule concerns both the codon/anticodon recognition level (first genetic code) and the tRNA/aaRS interaction level (second genetic code). In response to emergence of the CGG codon (that remains minor in *S.cerevisiae*), the gene corresponding to this relaxed tRNA would have been duplicated, leading to tRNA<sub>4</sub><sup>Arg</sup>, thus making tRNA<sub>4</sub><sup>Arg</sup> and tRNA<sub>4</sub><sup>Asp</sup> alloacceptor species. The further separation and improvement of the two specificities would have occurred by mutational

co-evolution of tRNA and aaRS, combined with epigenetic phenomena such as post-transcriptional modifications (22,43) and/or competition between the different partners (44,45).

This reduction of ambiguity in the second genetic code is supported by our *in vivo* experiments which show that the initially ambiguous tRNA<sub>4</sub><sup>Arg/C38/G73</sup> is not toxic for the cell but its aminoacylation properties are restricted to arginylation specificity. Although this may be due to the biased ratio of tRNA<sub>4</sub><sup>Arg/C38/G73</sup> (expressed from a centromeric plasmid) and tRNA<sub>4</sub><sup>Asp</sup> (expressed from 16 chromosomal copies of the same gene), there may also be competition events between AspRS and ArgRS for the same substrate in favor of arginylation. Further, aspartylation of tRNA<sub>4</sub><sup>Arg/C38/G73</sup> may be of low efficiency, because of post-transcriptional modifications introducing aspartate antideterminants into its structure. Also, the tRNA<sub>4</sub><sup>Arg</sup> variant, aminoacylated with aspartate, may not be recognized by the elongation factor EF-Tu and thus undergo deacylation (46). Alternatively, efficient aspartylation may occur, the mischarged tRNA being deacylated by autonomous proofreading proteins (47). According to the plateau theory (48), this would lead to faint levels of aspartylated

	tRNA <sub>GUC</sub> <sup>Asp</sup>	tRNA <sub>4</sub> <sup>Arg</sup>					
		CCU	ACG	UCU	CCG	UCG	GCG
		% $\frac{\alpha \beta v}{37 \ 38 / 73}$					
<i>Saccharomyces cerevisiae</i>	$\frac{3 \ 3 \ 4}{G \ C / G}$	49 $\frac{\bullet \circ \circ}{A \ A / G}$	47 $\frac{\bullet \bullet \circ}{A \ A / G}$	46 $\frac{\bullet \circ \circ}{A \ A / G}$	58 $\frac{\bullet \bullet \bullet}{G \ U / U}$	-	-
<i>Saccharomyces uvarum</i>	$\frac{3 \ 3 \ 4}{G \ C / G}$	-	49 $\frac{\bullet \bullet \circ}{A \ A / G}$	39 $\frac{\bullet \circ \circ}{A \ A / G}$	54 $\frac{\bullet \bullet \bullet}{G \ U / U}$	-	-
<i>Zygosaccharomyces rouxii</i>	$\frac{3 \ 3 \ 4}{G \ C / G}$	-	49 $\frac{\bullet \bullet \circ}{A \ A / G}$	38 $\frac{\bullet \circ \circ}{A \ A / U}$	56 $\frac{\bullet \bullet \bullet}{G \ U / U}$	-	-
<i>Candida glabrata</i>	$\frac{3 \ 3 \ 4}{G \ C / G}$	46 $\frac{\bullet \circ \circ}{A \ A / G}$	47 $\frac{\bullet \bullet \circ}{A \ A / G}$	40 $\frac{\bullet \circ \circ}{A \ A / G}$	54 $\frac{\bullet \bullet \bullet}{G \ U / U}$	-	-
<i>Kluyveromyces lactis</i>	$\frac{3 \ 3 \ 4}{G \ C / G}$	44 $\frac{\bullet \circ \circ}{A \ A / G}$	44 $\frac{\bullet \bullet \circ}{A \ A / G}$	38 $\frac{\bullet \circ \circ}{A \ A / G}$	60 $\frac{\bullet \bullet \bullet}{G \ U / U}$	-	-
<i>Debaryomyces hansenii</i>	$\frac{3 \ 3 \ 4}{G \ C / G}$	49 $\frac{\bullet \bullet \circ}{A \ A / U}$	46 $\frac{\bullet \bullet \circ}{A \ A / G}$	40 $\frac{\bullet \bullet \circ}{A \ A / U}$	40 $\frac{\circ \circ \circ}{G \ U / U}$	-	-
<i>Yarrowia lipolytica</i>	$\frac{3 \ 3 \ 4}{A \ C / G}$	53 $\frac{\bullet \circ \circ}{A \ A / G}$	49 $\frac{\bullet \circ \circ}{A \ A / G}$	42 $\frac{\bullet \bullet \circ}{A \ A / G}$	-	36 $\frac{\bullet \circ \circ}{G \ C / U}$	-
<i>Schizosaccharomyces pombe</i>	$\frac{3 \ 2 \ 4}{A \ C / G}$	47 $\frac{\bullet \bullet \circ}{A \ A / G}$	50 $\frac{\bullet \circ \circ}{G \ A / G}$	55 $\frac{\bullet \circ \circ}{A \ A / U}$	41 $\frac{\bullet \bullet \circ}{G \ C / G}$	41 $\frac{\bullet \circ \circ}{G \ C / G}$	-

legend  
 ● size conserved between tRNA<sup>Arg</sup> and tRNA<sup>Asp</sup>  
 ○ size non conserved

**Figure 6.** Characteristic features of tRNA<sup>Arg</sup> and tRNA<sup>Asp</sup> isoacceptors in various yeast species. tRNA genes were sought in eight yeast species (see Materials and Methods). Isoacceptors are named according to their anticodon triplets. In each case, the nucleotides at positions 37, 38 and 73 are given. Further, structural information on the nucleotide distribution in the subdomains  $\alpha$  and  $\beta$  of the D-loops as well as the size of the variable regions ( $v$ ) are indicated. Numbers indicate the percentage of identity of a given tRNA<sup>Arg</sup> with tRNA<sup>Asp</sup> in a same species. Along the left-hand side of the table, the phylogenetic link between the different yeast species is represented in a schematic diagram based on the rRNA sequences.

tRNA<sub>4</sub><sup>Arg/C38/G73</sup> that would escape detection by the method utilized. Finally, interaction of the mutant tRNA with other cellular compounds or structures could significantly influence its aminoacylation properties.

## OUTLOOK

One may speculate on the existence of an evolutionary link between the arginine and aspartate systems in other organisms. Systematic sequence and structure comparison of tRNA<sup>Arg</sup> with tRNA<sup>Asp</sup> from the available yeast genomes (*S.cerevisiae*, *S.uvarum*, *Z.rouxii*, *C.glabrata*, *K.lactis*, *D.hansenii*, *Y.lipolytica* and *S.pombe*) (49) show that a tRNA<sup>Arg</sup> isoacceptor, close in sequence and structure to tRNA<sup>Asp</sup>, can be found in the yeast species closest to *S.cerevisiae* (Figure 6). Indeed, a tRNA<sup>Arg</sup> with a CCG anticodon (tRNA<sub>4</sub><sup>Arg</sup> in *S.cerevisiae*), possessing a conserved number of elements in the  $\alpha$  and  $\beta$  parts of the D-loop as well as in the variable region, and further presenting nucleotides U38 and U73, can be found in these species. A tRNA<sup>Arg</sup> with sequence characteristics close to those of tRNA<sup>Asp</sup> has not so far been identified in bacteria or archaeobacteria. The evolutionary relationship between arginine- and aspartate-specific tRNAs thus seems to be restricted to a subset of yeast species.

The hypothesis of an evolutionary relation between tRNAs from different aminoacylation systems was explored here in the case of tRNA<sub>4</sub><sup>Arg</sup> and tRNA<sup>Asp</sup> within the yeast *S.cerevisiae*. The dendrogram suggests, according to the location of isolated tRNA genes far from clusters of additional genes from the same specificity, that a similar situation could exist for other aminoacylation systems, namely Thr- and Asn-specific tRNAs. It would also be interesting to explore cross-aminoacylation of aspartate and histidine systems, which are close neighbors in the evolutionary tree.

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