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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 $tRNA^{Asp}$ 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, $tRNA_4^{Arg}$. This tRNA has a sequence strikingly similar to that of $tRNA^{Asp}$ 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, $tRNA_4^{Arg}$ gains the highest aspartylation efficiency. Moreover, it is possible to convert $tRNA_4^{Arg}$ into an aspartate acceptor, as efficient as $tRNA^{Asp}$, 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 $tRNA_4^{Arg}$. The latent aspartate acceptor capacity in a contemporary $tRNA^{Arg}$ leads to the proposal of an evolutionary link between $tRNA_4^{Arg}$ and $tRNA^{Asp}$ 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, asparginyl-$tRNA^{Asn}$ is formed in a process involving mischarging of $tRNA^{Asn}$ 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 $tRNA^{Gln}$ by GluRS before being transamidated into glutamine (10). Similar situations exist for selenocysteine incorporation into proteins, with serine first being charged to $tRNA^{Sec}$ 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 $tRNA^{Asp}$ by ArgRS. The low but significant mischarging of yeast $tRNA^{Asp}$ by ArgRS discovered in the 1970s (7,20) can be dramatically improved when the tRNA is unmodified. Indeed, the $tRNA^{Asp}$ transcript can be charged by ArgRS as efficiently as the $tRNA_3^{Arg}$ isoacceptor (the four yeast $tRNA^{Arg}$ isoacceptor species with

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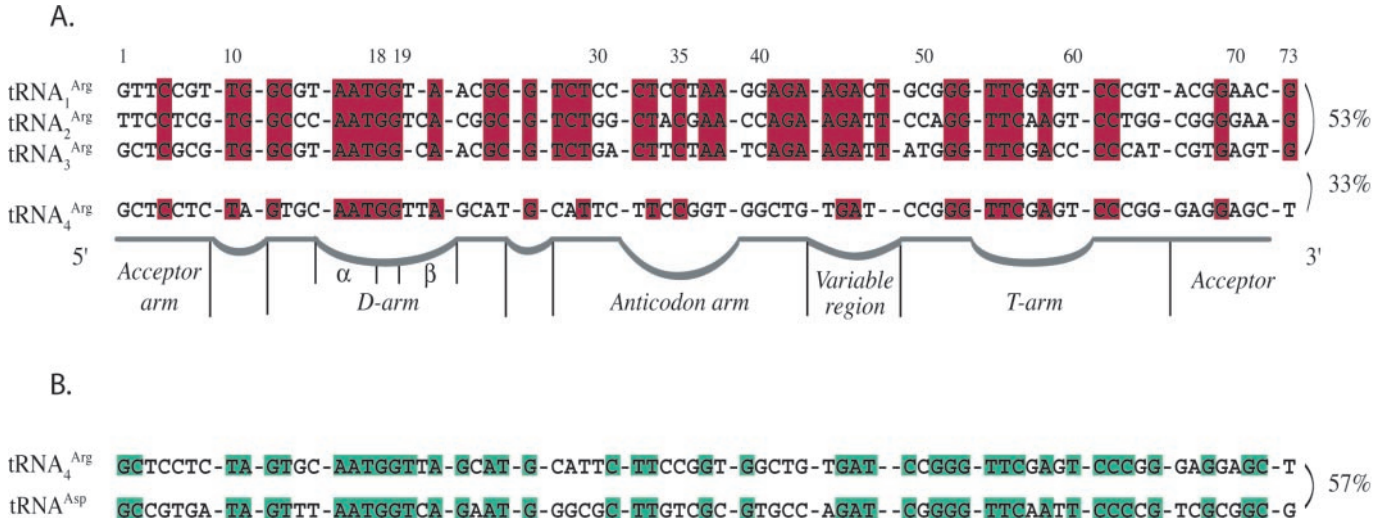

Figure 1. Sequence comparison and tertiary structural elements of yeast aspartate and arginine isoacceptor tRNAs. (**A**) $tRNA^{Arg}$ isoacceptors 1, 2 and 3 (the three major species decoding 96.1% of the arginine codons) show 53% identity. Isoacceptor 4 is distinguished from the other three in sharing 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 $tRNA_4^{Arg}$ and the single $tRNA^{Asp}$ isoacceptor indicates 57% identity. These two sequences share a same length in their variable region and in the α and β domains of the D-loop.

anticodons CCU, ACG, UCU and CCG are termed as $tRNA_1^{Arg}$, $tRNA_2^{Arg}$, $tRNA_3^{Arg}$ and $tRNA_4^{Arg}$, respectively), and therefore can be considered as an additional arginine acceptor (21). The protection of native $tRNA^{Asp}$ against efficient recognition by ArgRS is due to the N^1 methyl group on G37 antideterminant activity (22). Notably, the misarginylation potential of $tRNA^{Asp}$ is not mediated by mimics of the classical arginine identity elements present in $tRNA_3^{Arg}$, but by an alternative set of determinants (C36 and G37, instead of C35 and U/G36) (19).

The reverse situation, recognition of $tRNA^{Arg}$ by AspRS, has been less studied. Potential aspartylation properties of the four $tRNA^{Arg}$ 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 $tRNA^{Asp}$ gene and revealed the existence of a single-copy gene of a minor $tRNA_4^{Arg}$, in addition to three already known $tRNA^{Arg}$ isoacceptors. This minor tRNA is vital for the cell (25). Sequence comparisons indicate ~53% identity among $tRNA_1^{Arg}$, $tRNA_2^{Arg}$ and $tRNA_3^{Arg}$, and reveal a divergent $tRNA_4^{Arg}$ sequence sharing only 33% of its residues with the three other isoacceptors (Figure 1A). Interestingly, however, $tRNA_4^{Arg}$ shares 57% of its residues with $tRNA^{Asp}$ (Figure 1B) and conversely $tRNA^{Asp}$ does not show significant sequence similarity to other arginine isoacceptors (46–49%). This structural relationship between $tRNA_4^{Arg}$ and $tRNA^{Asp}$ is also related to the size of the α/β regions in their D-loops ($\alpha = \beta = 3$) and that of their variable region ($v = 4$), different in the three other $tRNA^{Arg}$ isoacceptors (Figure 1A and B). In summary, $tRNA_4^{Arg}$ sequence and structure more closely resemble those of $tRNA^{Asp}$ than those of the other three $tRNA^{Arg}$ 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 $tRNA_4^{Arg}$, is located at a long distance, closer to the sequence of $tRNA^{Asp}$. This suggests an evolutionary link between $tRNA_4^{Arg}$ and $tRNA^{Asp}$.

The aim of the present study is to explore whether the sequence relationship between $tRNA_4^{Arg}$ and $tRNA^{Asp}$ 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 $tRNA_4^{Arg}$ into a substrate for AspRS as efficient as homologous $tRNA^{Asp}$ 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 $tRNA^{Asp}$ and $tRNA_3^{Arg}$ 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 Thermohybaid, 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-^{3}H]aspartic acid

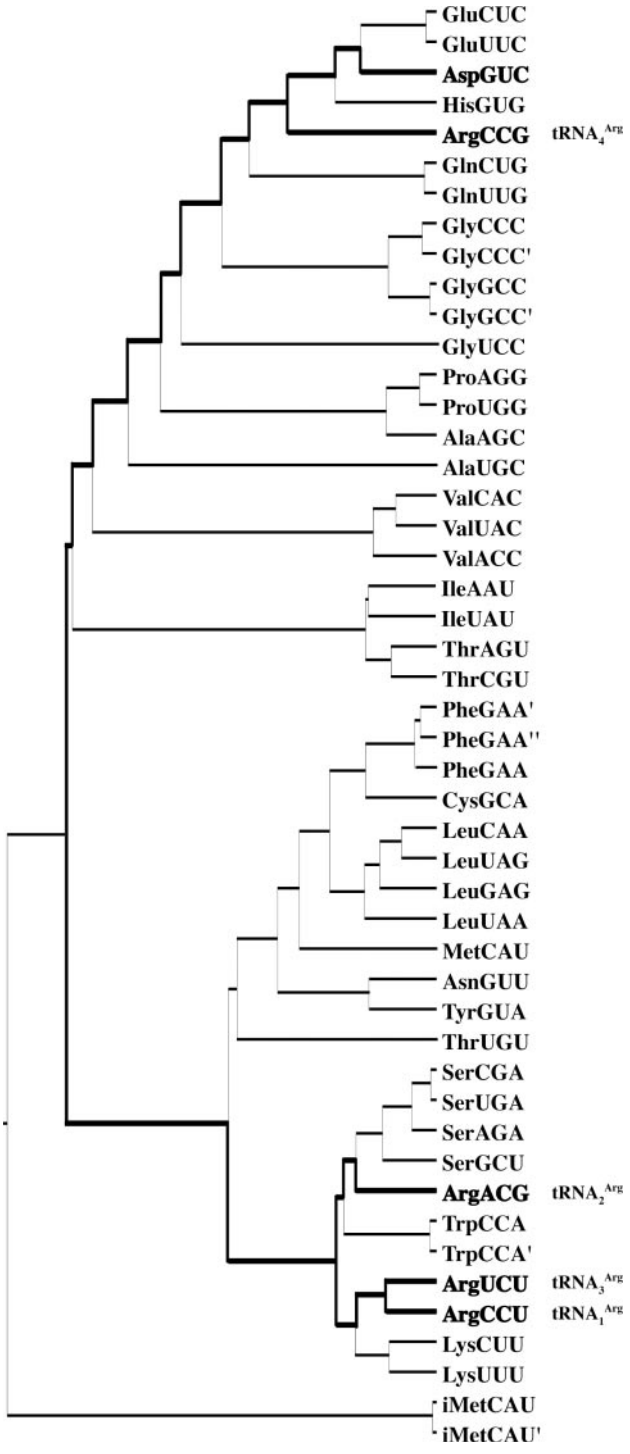

Figure 2. Sequence distances in the 48 yeast tRNA genes highlight proximity between $tRNA^{Asp}$ and $tRNA_4^{Arg}$. The dendrogram was established with the ClustalV program from MegAlign (Lasergene from DNAstar). Genes for $tRNA^{Arg}$ and $tRNA^{Asp}$ are highlighted as well as either short or long 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), [L-^{3}H]arginine (41 Ci/mmol), [γ-^{32}P]dATP and Hybond-XL nylon membrane were from Amersham. Yeast strain YAL5 n [*ura3-52 lys2-801*^{am} *trp1-Δ63 his3-Δ200 leu2-Δ1 ade2-Δ450 ade3-Δ1483*, $tRNA_4^{Arg}$*::HIS3*, pAL5 ($tRNA_4^{Arg+}$, Ura^+, $Ade3^+$)] and plasmid pRS314-$tRNA_4^{Arg}$ ($tRNA_4^{Arg+}$, Trp^+) containing the wild-type $tRNA_4^{Arg}$ 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 $tRNA_3^{Arg}$ and $tRNA^{Asp}$, or with a higher concentration of $MgCl_2$ (30 mM versus 22 mM) for tRNAs cloned with an upstream ribozyme sequence (30). A BstNI site (or NsiI for $tRNA_2^{Arg}$ and for variant $tRNA_2^{Arg/Asp}$) 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 $MgCl_2$, 10 mM ATP, 2.5 mM glutathione, 50 μM [^{3}H]arginine (74 GBq/mmol) and adequate amounts of transcript (50 nM to 6 μM) and yeast ArgRS (2–730 nM). Aspartylation conditions were 50 mM HEPES–NaOH pH 7.5, 30 mM KCl, 15 mM $MgCl_2$, 5 mM ATP, 32 μM [^{3}H]aspartic acid (208 GBq/mmol), and adequate amounts of transcript (20 nM to 6 μM) and yeast AspRS (15 nM to 1 μM). 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 [$MgCl_2$]/[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 μM 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-$tRNA_4^{Arg}$ was converted into pRS314-$tRNA_4^{Arg/C38/G73}$ containing mutant $tRNA_4^{Arg/C38/G73}$ gene using a QuikChange™ 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 μg of pRS314-$tRNA_4^{Arg/C38/G73}$ was used to transform the yeast red phenotype YAL5 strain, previously disrupted at $tRNA_4^{Arg}$ genomic sequence by the mutant gene Δ$tRNA_4^{Arg}$*::HIS* and rescued by the plasmid pAL5 ($tRNA_4^{Arg+}$, Ura^+,

Ade3$^+$) (25), on minimal medium supplemented with adenine (limiting concentration of 2 μg ml^{-1}), uracil (20 μg ml^{-1}), lysine (30 μg ml^{-1}) and leucine (60 μg ml^{-1}) (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*$_4^{Arg/C38/G73}$ and so express only the double mutant tRNA$_4^{Arg/C38/G73}$.

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_{700} = 2) or during exponential phase (OD_{700} = 0.7). Extraction of total RNA was performed on ice with acidic phenol (saturated with 0.3 M sodium acetate and 10 mM Na_2EDTA, 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′-^{32}P]oligo-labeled nucleotides. The probe for tRNA$_4^{Arg}$ was 5′-CTCGA-ACCCGGATCACAGCC, annealing to positions 39–59; the probe for 5S rRNA was 5′-ACCCACTACACTACTCGGT-CAGGCTCTTAC; and for tRNA$_{UAA}^{Leu}$ was 5′-GGATGCGA-GGTTCGAACTCGCGCGG. 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^{Arg} (CCU, ACG, UCU, CCG, UCG, GCG) and tRNA^{Asp} (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^{Asp} was compared with each tRNA^{Arg} 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^{Arg} isoacceptor species

Aspartylation properties of the four *in vitro* transcribed tRNA^{Arg} were tested under standard experimental conditions (5 mM ATP and 15 mM $MgCl_2$) as well as under less stringent conditions (0.5 mM ATP and 15 mM $MgCl_2$). 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^{Asp} is efficiently aspartylated (charging level up to 75%), the four yeast tRNA^{Arg} 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^{Arg} isoacceptors. Although tRNA$_2^{Arg}$ and tRNA$_3^{Arg}$ display a large decrease in catalytic efficiency (≥250 000-fold), tRNA$_1^{Arg}$ and tRNA$_4^{Arg}$ cause somewhat less drastic decrease (67 000-fold).

Under misaminoacylation conditions ([$MgCl_2$]/[ATP] = 30), the four tRNA^{Arg} become more efficiently misaspartylated. Most noticeable is the gain in aminoacylation efficiency of tRNA$_4^{Arg}$. Although tRNA$_1^{Arg}$, tRNA$_2^{Arg}$ and tRNA$_3^{Arg}$ remain charged to <1%, aspartylation of tRNA$_4^{Arg}$ reaches a level of 9%. When compared to tRNA^{Asp}, losses in aminoacylation reach 20 000- to 100 000-fold for tRNA$_1^{Arg}$, tRNA$_2^{Arg}$ or tRNA$_3^{Arg}$, respectively. For tRNA$_4^{Arg}$ 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$_3^{Arg}$, 3.3-fold for tRNA$_1^{Arg}$, 12.5-fold for tRNA$_2^{Arg}$ and 30.5-fold for tRNA$_4^{Arg}$. 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$_4^{Arg}$ clearly distinguishes itself in aspartylation activity from the other tRNA^{Arg} isoacceptors.

tRNA$_4^{Arg}$ 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^{Arg} isoacceptors and yeast tRNA^{Asp}

Transcript	Standard conditions[a] (5 mM ATP and 15 mM $MgCl_2$)			'Permissive' conditions (0.5 mM ATP and 15 mM $MgCl_2$)		
	Charging level (%)	k_{cat}/K_M (10^{-3} s^{-1} nM^{-1})	L (fold)[b]	Charging level (%)	k_{cat}/K_M (10^{-3} s^{-1} nM^{-1})	L (fold)[b]
tRNA^{Asp}	75	0.5	1	100	1.0	1
tRNA$_1^{Arg}$	<1	7.5×10^{-6}[c]	67 000	<1	2.5×10^{-5}[c]	20 000
tRNA$_2^{Arg}$	<1	2.0×10^{-6}[c]	250 000	<1	2.5×10^{-5}[c]	20 000
tRNA$_3^{Arg}$	<1	nd	>250 000	<1	5.0×10^{-6}[c]	100 000
tRNA$_4^{Arg}$	<1	7.5×10^{-6}[c]	67 000	9	2.3×10^{-4}[c]	2200

nd, too low to detect.
[a]Values from Fender *et al.* (23).
[b]L stands for the relative loss in aminoacylation efficiency considering tRNA^{Asp} as the reference [for the definition of L, see (50)].
[c]Determined according to the method described in (3).

set was transplanted into each of the four $tRNA^{Arg}$ 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. $tRNA_1^{Arg/Asp}$, $tRNA_2^{Arg/Asp}$ and $tRNA_3^{Arg/Asp}$ almost completely lost their natural arginylation ability (charging levels <10%). Loss of catalytic efficiency compared to wild-type $tRNA^{Asp}$ varies from 7400-fold to more than 10 000-fold. On the other hand, $tRNA_4^{Arg/Asp}$ retains the ability to be efficiently arginylated with a charging level of 80% and a

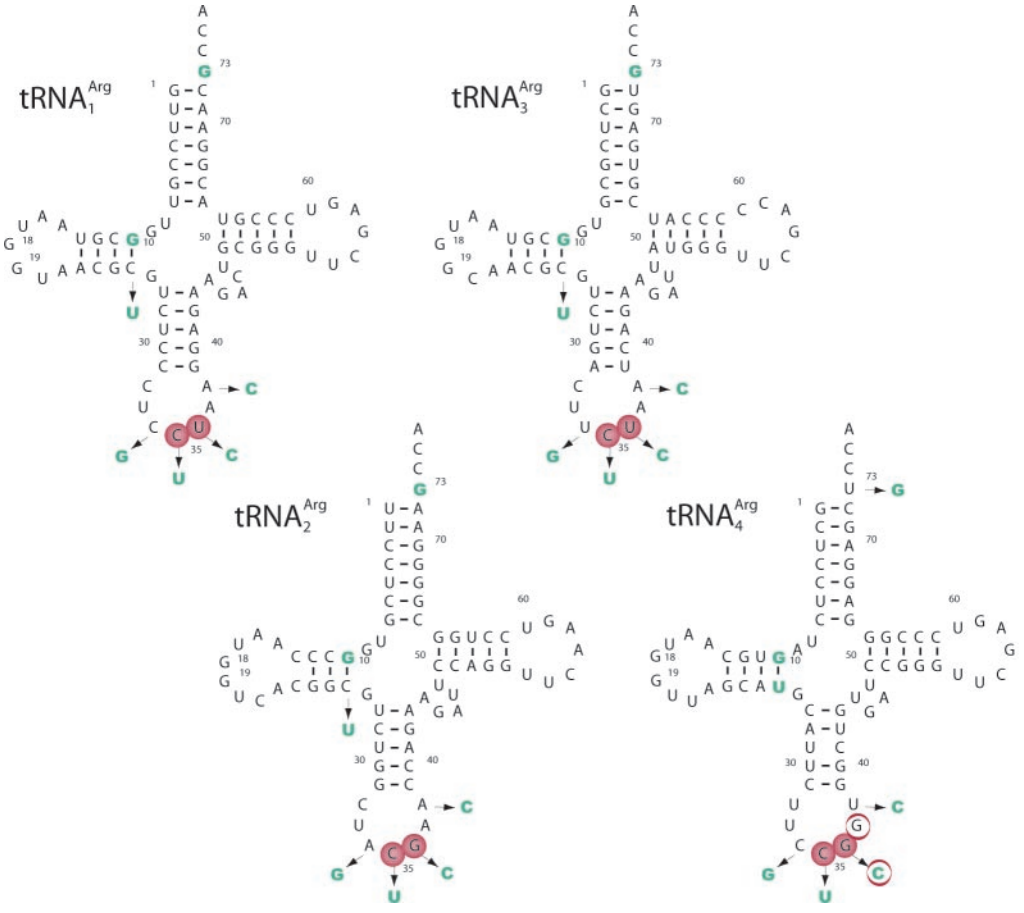

Figure 3. Cloverleaf sequences of the four yeast $tRNA^{Arg}$ isoacceptors. Full red dots highlight the set of arginine identity elements experimentally determined for $tRNA_3^{Arg}$ (19). Insertion of the aspartate identity set (17,18) indicated in green, generates an alternative arginine identity set (19) in $tRNA_4^{Arg}$ (nucleotides in red circles).

Table 2. Arginylation and aspartylation of *in vitro* transcribed yeast $tRNA^{Arg}$ isoacceptors into which the whole set of aspartate identity elements was transplanted

Transcript	Charging level (%)	k_{cat} (s^{-1})	K_M (nM)	k_{cat}/K_M (10^{-3} s^{-1} nM^{-1})	L (fold)[a]
Arginylation					
$tRNA^{Asp}$	100	0.3	220	1.4	1
$tRNA_1^{Arg/Asp}$	<5	nd	nd	1.4×10^{-4}[b]	10 000
$tRNA_2^{Arg/Asp}$	<1	nd	nd	nd	>10 000
$tRNA_3^{Arg/Asp}$	10	8.9×10^{-4}	4680	1.9×10^{-4}	7400
$tRNA_4^{Arg/Asp}$	80	0.2	190	1.1	1
Aspartylation[c]					
$tRNA^{Asp}$	75	0.03	60	0.5	1
$tRNA_1^{Arg/Asp}$	80	0.03	130	0.2	2.5
$tRNA_2^{Arg/Asp}$	<10	9.2×10^{-5}	3225	3.0×10^{-5}	16 700
$tRNA_3^{Arg/Asp}$	<10	76×10^{-5}	1460	52×10^{-5}	960
$tRNA_4^{Arg/Asp}$	80	0.06	130	0.5	1

nd, too low to detect. Aminoacylation reactions were performed under 'standard' conditions.
[a]L stands for relative loss in aminoacylation efficiency considering $tRNA^{Asp}$ as the reference.
[b]Determined according to the method described in (3).
[c]Values from (23).

Table 3. Aspartylation and arginylation of *in vitro* transcribed yeast $tRNA_4^{Arg}$ and variants

Transcript	Charging level (%)	k_{cat} (s^{-1})	K_M (nM)	k_{cat}/K_M (10^{-3} s^{-1} nM^{-1})	L (fold)[a]
Aspartylation					
$tRNA^{Asp}$	75	0.03	60	0.5	1
$tRNA_4^{Arg}$	<1	nd	nd	7.5×10^{-6}[b]	67 000
$tRNA_4^{Arg/C38}$	13	0.0003	290	0.001	500
$tRNA_4^{Arg/G73}$	72	0.004	300	0.01	50
$tRNA_4^{Arg/C38/G73}$	98	0.07	440	0.2	2.5
Arginylation					
$tRNA_4^{Arg}$	90	0.5	470	1.1	1
$tRNA_4^{Arg/C38}$	88	0.2	20	10.0	0.1
$tRNA_4^{Arg/G73}$	74	0.2	920	0.2	5.5
$tRNA_4^{Arg/C38/G73}$	65	0.3	190	1.6	0.7

nd, too low to detect. Aminoacylation reactions were performed under 'standard' conditions.
[a]L stands for relative loss in aminoacylation efficiency taking $tRNA^{Asp}$ (upper part) or $tRNA_4^{Arg}$ (lower part) as the references.
[b]Determined according to the method described in (3).

catalytic efficiency comparable to that of $tRNA^{Asp}$ ($L = 1$). Thus, in addition to being the best transplanted substrate for AspRS (23), $tRNA_4^{Arg/Asp}$ has unusual arginylation properties compared to the other arginine isoacceptors transplanted with aspartate identity elements.

Two mutations are sufficient to convert $tRNA_4^{Arg}$ into an efficient aspartate acceptor

The possibility that $tRNA_4^{Arg}$ could be converted into an efficient aspartate acceptor by a small number of mutations was explored. Three variants of $tRNA_4^{Arg}$ have been prepared. Two mutants had substitutions U38→C38 ($tRNA_4^{Arg/C38}$) or U73→G73 ($tRNA_4^{Arg/G73}$). The double mutant $tRNA_4^{Arg/C38/G73}$ 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 $tRNA_4^{Arg}$ with charging levels varying between 13 and 98%. Affinity of AspRS for each of the three variants estimated by K_M is very similar (290–440 nM). Strong differences in catalytic efficicency was observed with k_{cat} of 0.3×10^{-3} and 4×10^{-3} s^{-1} for the single mutants $tRNA_4^{Arg/C38}$ and $tRNA_4^{Arg/G73}$, respectively, and a large increase to 70×10^{-3} s^{-1} for the double mutant $tRNA_4^{Arg/C38/G73}$. Thus, each single mutation causes aspartylation capacity to increase significantly compared to wild-type $tRNA_4^{Arg}$. 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 $tRNA_4^{Arg/C38/G73}$ becomes only 2.5-fold less efficiently aspartylated as $tRNA^{Asp}$ with a 26 800-fold gain compared to wild-type $tRNA_4^{Arg}$.

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_{cat} are similar to the four transcripts but Michaelis–Menten constants K_M are different. Although for variant $tRNA_4^{Arg/G73}$ and $tRNA_4^{Arg/C38/G73}$ K_M is with a factor 2 of that of $tRNA_4^{Arg}$, this parameter is 23.5-fold lower than that for $tRNA_4^{Arg/C38}$. 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 $tRNA_4^{Arg}$ variant in which only two positions have been modified, $tRNA_4^{Arg/C38/G73}$, which remains a very efficient substrate for arginylation but which also becomes an aspartylation substrate as efficient as wild-type $tRNA^{Asp}$.

In vivo, the double mutated $tRNA_4^{Arg/C38/G73}$ is only arginylated

To investigate the effect of the dual arginine and aspartate acceptance of $tRNA_4^{Arg/C38/G73}$ on growth, we used a knockout strain deprived of the minor $tRNA_4^{Arg}$ 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 $tRNA_4^{Arg}$ genes by plasmid shuffling to exchange the wild-type gene with the mutated gene. A successful exchange means that the $tRNA_4^{Arg}$ 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 $tRNA_4^{Arg/C38/G73}$ gene carried in a pRS314 vector. The shuffle of pAL5 by pRS314-$tRNA_4^{Arg/C38/G73}$ was followed by a loss of red pigmentation (colonies appear white/red sectored, then white) due to loss of *ADE3* (see Material and Methods). Therefore, $tRNA_4^{Arg/C38/G73}$ encoded by pRS314-$tRNA_4^{Arg/C38/G73}$ is capable of rescuing the $tRNA_4^{Arg}$-knockout strain, which means that this tRNA is functional in translation *in vivo*.

tRNA extracted from the $tRNA_4^{Arg}$-knockout strain containing exclusively $tRNA_4^{Arg/C38/G73}$, was analyzed using northern blot under acidic conditions. Figure 4 compares the hybridization patterns of $tRNA_4^{Arg}$ and mutant $tRNA_4^{Arg/C38/G73}$ to $tRNA^{Asp}$ signals. The addition of an arginine residue to the tRNA molecule produces a consequent shift on the $tRNA^{Arg}$ migration (lanes 1 and 3), whereas that of an aspartate residue to $tRNA^{Asp}$ only produces less of a shift (lane 5). No signal corresponding to aspartyl-$tRNA_4^{Arg/C38/G73}$ is present in lane 3, indicating that significant aspartyl-$tRNA^{Arg}$ formation did not occur *in vivo*. However, quantification of the signals reveals that the arginyl-$tRNA^{Arg}$ levels were significantly changed

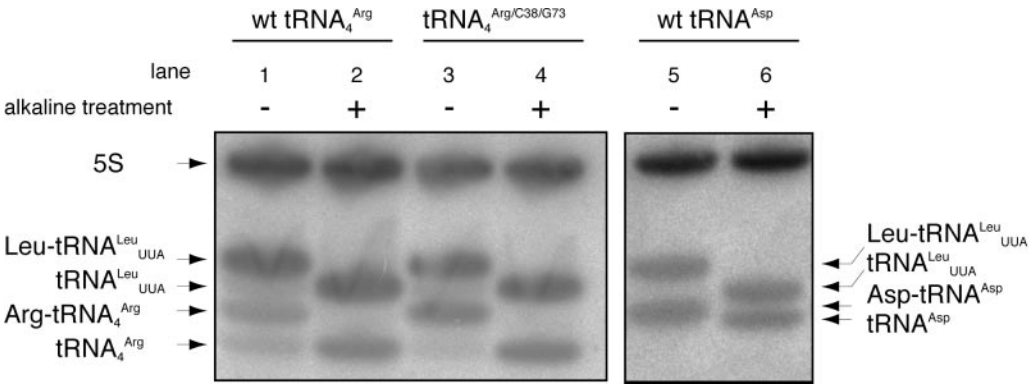

Figure 4. Northern-blot analysis of tRNA samples extracted from yeast cells under acidic conditions. Experimental procedure is described in Materials and Methods. Levels of aminoacylation of $tRNA_4^{Arg}$ and $tRNA_4^{Arg/C38/G73}$ were determined by measurement of the ratio of acylated (− lanes) and deacylated tRNA (+ lanes, obtained by alkaline treatment of the RNA extracts). The blot was probed with ^{32}P-labeled oligonucleotides complementary to 5S RNA (control of RNA content), to $tRNA^{Leu}$ (control of the acidic extraction procedure) and to $tRNA_4^{Arg}$. Lanes 1 and 2, wild-type $tRNA_4^{Arg}$; and lanes 3 and 4, mutated $tRNA_4^{Arg/C38/G73}$. The second blot (lanes 5 and 6) was probed with a $tRNA^{Asp}$-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 $tRNA_4^{Arg}$ is usually ~70%, it becomes 85% for mutant $tRNA_4^{Arg/C38/G73}$ in the yeast cells (a reproducible observation for several independent experiments). An increased steady-state level in arginylation of $tRNA_4^{Arg/C38/G73}$ 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 $tRNA_4^{Arg}$ to $tRNA^{Asp}$ correlates with functional relationship

Sequencing the complete yeast genome revealed the existence of a fourth $tRNA^{Arg}$ isoacceptor, $tRNA_4^{Arg}$. Sequence comparison and multiple sequence alignment emphasized the striking similarity of $tRNA_4^{Arg}$ to $tRNA^{Asp}$. Thus, these two tRNAs were predicted to be evolutionarily related since they are clustered far from the three other $tRNA^{Arg}$ 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 α and β 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 $tRNA^{Arg}$ and $tRNA^{Asp}$ *in vitro* transcripts demonstrates that $tRNA_4^{Arg}$ is also functionally distant from the three other arginine acceptors and close to $tRNA^{Asp}$. 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, $tRNA_4^{Arg}$ is the acceptor which becomes best aspartylated under permissive conditions (30.5 fold better) although it still remains 2200-fold less efficiently charged than $tRNA^{Asp}$. (ii) When the full set of aspartate identity elements is transplanted into the $tRNA^{Arg}$ isoacceptors, only $tRNA_4^{Arg}$ 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 $tRNA_4^{Arg}$. 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 $tRNA_4^{Arg}$ molecule expresses the alternate arginine identity set [C36G37 initially found within *in vitro* transcribed $tRNA^{Asp}$, (19)] that results from the combination of residue G37, naturally present in $tRNA_4^{Arg}$ but absent within the three other $tRNA^{Arg}$, 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 $tRNA_1^{Arg}$, $tRNA_2^{Arg}$ and $tRNA_3^{Arg}$. (iii) It was possible to convert $tRNA_4^{Arg}$ with a minimal number of mutations into an efficient aspartate accepting tRNA.

Despite its significant resemblance to $tRNA^{Asp}$, wild-type $tRNA_4^{Arg}$ 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 $tRNA_4^{Arg}$ to be mutated have to be peculiar to this tRNA, i.e. absent from $tRNA^{Asp}$ and from the three other $tRNA^{Arg}$. Two nucleotides, U73 and U38, were found to be good candidates (Figure 5). Position 73 is crucial for $tRNA^{Asp}$ 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 $tRNA_4^{Arg}$. Notably, G73 present in $tRNA_1^{Arg}$, $tRNA_2^{Arg}$ and $tRNA_3^{Arg}$, 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 $tRNA_4^{Arg}$. The three other arginine isoacceptor tRNAs display the doublet A37A38 within the anticodon loop, while $tRNA_4^{Arg}$ possesses the sequence G37U38. The residue G37 is found in $tRNA^{Asp}$ but followed by C38, which contributes to a low extend to aspartate identity [5-fold effect; (18)]. Upon substitution, these residues are predicted to enable aspartylation of $tRNA_4^{Arg}$. Both substitutions were beneficial for $tRNA_4^{Arg}$ aspartylation. Replacement of either U73 or U38 in $tRNA_4^{Arg}$ by G73 or C38 leads to significant increase in catalytic efficiency (1340- and 134-fold, respectively). Combination of these two mutations converts

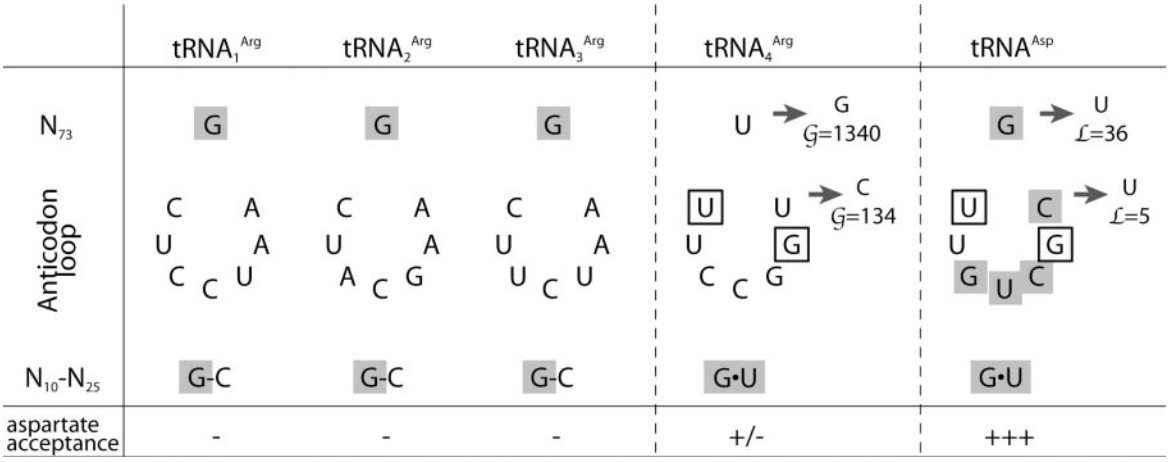

Figure 5. Yeast aspartate identity elements in the four tRNA^{Arg} isoacceptors. Nucleotides at position 73 (discriminator base), the anticodon loop and 10–25 bp are indicated. For comparison, the sequence of the single tRNA^{Asp} isoacceptor is also indicated. Aspartate identity elements are highlighted with gray squares. Note the absence of aspartate determinants from the anticodon loop of each of the four tRNA^{Arg} isoacceptors. tRNA$_4^{Arg}$ shares two nucleotides with tRNA^{Asp} (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$_4^{Arg}$ and tRNA^{Asp} are indicated.

tRNA$_4^{Arg}$ into a substrate for AspRS almost as efficient as tRNA^{Asp} (tRNA$_4^{Arg/C38/G73}$ is only 2.5-fold less efficiently aspartylated than tRNA^{Asp}).

Notably, in addition to gaining aspartylation specificity, the double mutant tRNA$_4^{Arg/C38/G73}$ 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$_3^{Arg}$; (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$_4^{Arg}$ 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$_4^{Arg}$.

Cryptic aspartate identity elements embedded in tRNA$_4^{Arg}$

tRNA$_4^{Arg}$ 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$_1^{Arg}$, tRNA$_2^{Arg}$ and tRNA$_3^{Arg}$ possess the major G73 aspartate identity element, tRNA$_4^{Arg}$ displays only the minor G10•U25 element (17). This was a first argument illuminating the possibility that cryptic aspartate elements are embedded in tRNA$_4^{Arg}$. Proof came from the double mutant tRNA$_4^{Arg/C38/G73}$, 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$_4^{Arg}$. Contribution to aspartylation efficiency of residues G73 and C38 have been estimated previously by studies performed on tRNA^{Asp}. 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$_4^{Arg}$. Strikingly, the double variant tRNA$_4^{Arg/C38/G73}$ 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$_4^{Arg/C38/G73}$ results from an alternative set of identity elements from that established within the tRNA^{Asp} framework. These cryptic elements still have to be deciphered.

Sequence and functional similarities of tRNA$_4^{Arg}$ and tRNA^{Asp} reveal an evolutionary relationship

We have uncovered remnant aspartate acceptor properties within a contemporary arginine-specific tRNA and propose an evolutionary linkage between *tRNA*$_4^{Arg}$ and *tRNA*^{Asp} 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$_4^{Arg}$. Indeed, U73 and U38 are believed to play a major role during the evolutionary establishment of the restricted arginine specificity of tRNA$_4^{Arg}$, and to have been introduced during evolution to hide the peculiar aspartylation potential of tRNA$_4^{Arg}$. Mutation of these nucleotides allowed us to artificially turn back evolutionary, so that tRNA$_4^{Arg/C38/G73}$ would appear to be an intermediate in the history of tRNA$_4^{Arg}$. 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 $tRNA_4^{Arg}$, thus making $tRNA_4^{Arg}$ and $tRNA^{Asp}$ 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 $tRNA_4^{Arg/C38/G73}$ 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 $tRNA_4^{Arg/C38/G73}$ (expressed from a centromeric plasmid) and $tRNA^{Asp}$ (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 $tRNA_4^{Arg/C38/G73}$ may be of low efficiency, because of post-transcriptional modifications introducing aspartate antideterminants into its structure. Also, the $tRNA^{Arg}$ 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

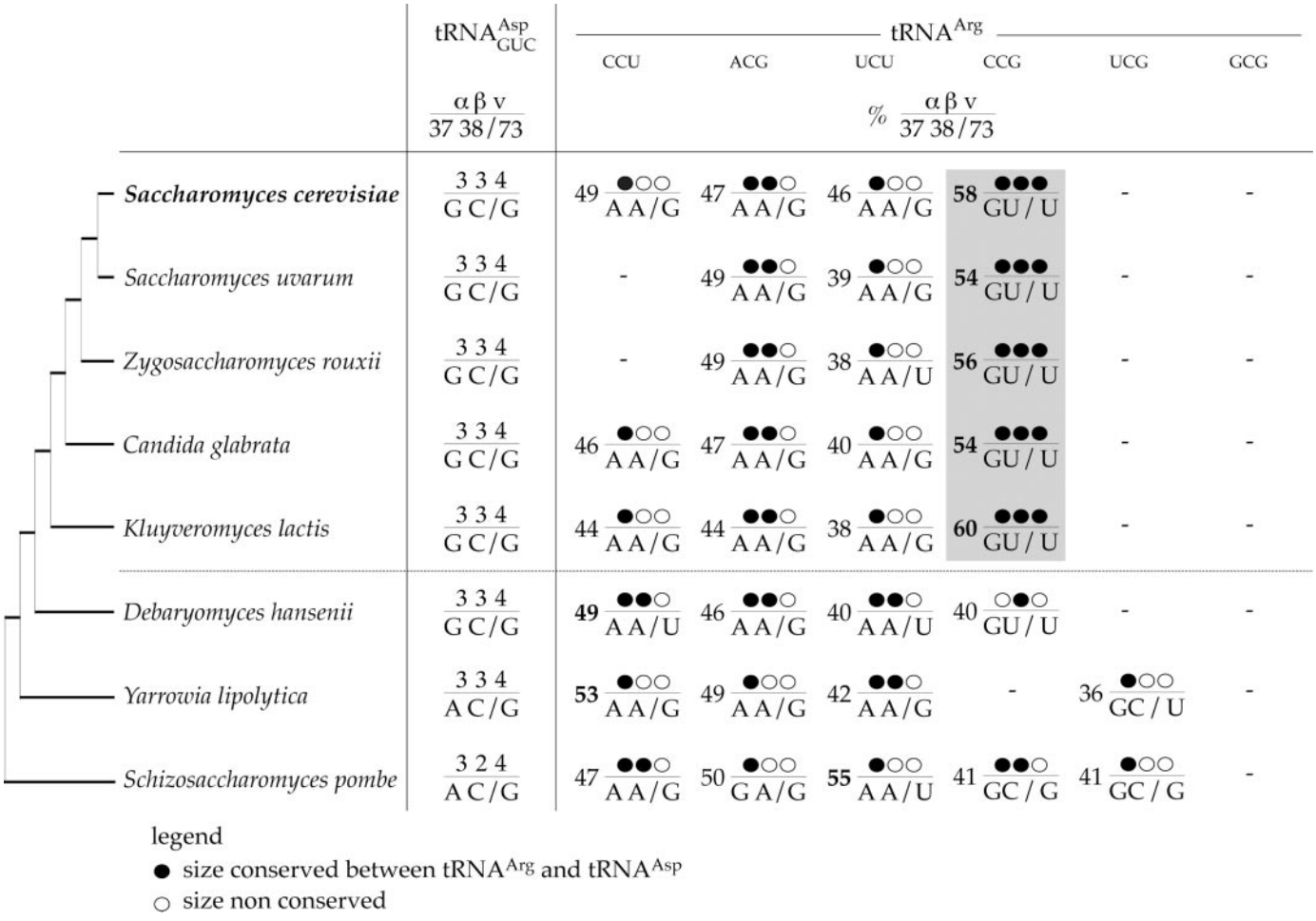

Figure 6. Characteristic features of $tRNA^{Arg}$ and $tRNA^{Asp}$ isoacceptors in various yeast species. tRNA genes were sought in eight yeast species (see Materials and Methods). Isoacceptors are named according to their anticodon triplets. In each case, the nucleotides at positions 37, 38 and 73 are given. Further, structural information on the nucleotide distribution in the subdomains α and β 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 $tRNA^{Arg}$ with $tRNA^{Asp}$ in a same species. Along the left-hand side of the table, the pylogenetic link between the different yeast species is represented in a schematic diagram based on the rRNA sequences.

$tRNA_4^{Arg/C38/G73}$ 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 $tRNA^{Arg}$ with $tRNA^{Asp}$ 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 $tRNA^{Arg}$ isoacceptor, close in sequence and structure to $tRNA^{Asp}$, can be found in the yeast species closest to *S.cerevisiae* (Figure 6). Indeed, a $tRNA^{Arg}$ with a CCG anticodon ($tRNA_4^{Arg}$ in *S.cerevisiae*), possessing a conserved number of elements in the α and β 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 $tRNA^{Arg}$ with sequence characteristics close to those of $tRNA^{Asp}$ has not so far been identified in bacteria or archaebacteria. 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 $tRNA_4^{Arg}$ and $tRNA^{Asp}$ 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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