Identity of tRNA for Yeast Tyrosyl-tRNA Synthetase: Tyrosylation Is More Sensitive to Identity Nucleotides Than to Structural Features†

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ABSTRACT: The specific aminoacylation of tRNA by yeast tyrosyl-tRNA synthetase does not rely on the presence of modified residues in $tRNA^{Tyr}$, although such residues stabilize its structure. Thus, the major tyrosine identity determinants were searched by the in vitro approach using unmodified transcripts produced by T7 RNA polymerase. On the basis of the tyrosylation efficiency of tRNA variants, the strongest determinants are base pair C1−G72 and discriminator residue A73 (the 5′-phosphoryl group on C1, however, is unimportant for tyrosylation). The three anticodon bases G34, U35, and A36 contribute also to the tyrosine identity, but to a lesser extent, with G34 having the most pronounced effect. Mutation of the GUA tyrosine anticodon into a CAU methionine anticodon, however, leads to a loss of tyrosylation efficiency similar to that obtained after mutation of the C1−G72 or A73 determinants. Transplantation of the six determinants into four different tRNA frameworks and activity assays on heterologous *Escherichia coli* and *Methanococcus jannaschii* $tRNA^{Tyr}$ confirmed the completeness of the tyrosine set and the eukaryotic character of the C1−G72 base pair. On the other hand, it was found that tyrosine identity in yeast does not rely on fine architectural features of the tRNA, in particular the size and sequence of the D-loop. Noticeable, yeast TyrRS efficiently charges a variant of *E. coli* $tRNA^{Tyr}$ with a large extra-region provided its G1−C72 base pair is changed to a C1−G72 base pair. Finally, tyrosylation activity is compatible with a +1 shift of the anticodon in the 3′-direction but is strongly inhibited if this shift occurs in the opposite 5′-direction.

The understanding of accurate genetic code expression at the translational level requires a precise knowledge of the tRNA[1] identity rules and their idiosyncrasies in evolution (*1*). Major identity elements have been deciphered for the 20 amino acid specificities in *Escherichia coli*, but in the case of the yeast *Saccharomyces cerevisiae*, the model organism for lower eukaryotes, information is less complete. This paper concerns establishment of the complete tyrosine identity set in tRNA for its charging by yeast tyrosyl-tRNA synthetase (TyrRS). Indeed this set is yet not completely deciphered and not well understood for its evolutionary relationship with tyrosine identities in other organisms. This may appear surprising when considering the astonishing structural and functional features of tyrosine systems but is explained by the technical difficulties only recently solved for the production of the needed $tRNA^{Tyr}$ mutants. Indeed, these mutants had to be produced by self-cleavage of 5′-extended RNAs because of the unfavorable 5′-sequence of $tRNA^{Tyr}$ for transcription by T7 RNA polymerase (*2*).

Beside their classical function in protein synthesis, TyrRSs and $tRNA^{Tyr}$ or their mimics are involved in other cellular processes. TyrRSs participate in group I intron splicing in *Neurospora crassa* mitochondria (*3*) and in cellular signaling in humans (*4*). On the RNA side, tyrosylable tRNA-like structures have been found in RNA genomes of plant viruses (reviewed in refs *5* and *6*) and $tRNA^{Tyr}$ mimicry was described in the catalytic core of the *Neurospora* introns (*7*). From another point of view, investigations on TyrRSs were at the forefront in crystallography of synthetases (*8*) and in protein engineering (*9*). Similarly, understanding of suppression largely relies on the pioneering genetic studies of $tRNA^{Tyr}$ suppressors (reviewed in ref *10*) and RNA microsurgery technologies partly originated from studies on $tRNA^{Tyr}$ (*11*, *12*). As to the sequences of tyrosine specific tRNAs, they have peculiarities which are unique among tRNAs (*13*). The most prominent one concerns the variable region, which is of small size in eukaryotic $tRNA^{Tyr}$ species (Figure 1A) and is constituted by a stem/loop structure in eubacterial species (Figure 1B).

In *E. coli*, tyrosine identity was investigated by both in vivo and in vitro methods and the importance of the anticodon loop, with the explicit participation of U35, the discriminator base A73, and the long variable region was emphasized (*14*−*17*). In yeast, the involvement in tyrosine identity of anticodon residues G34 and Ψ35 was first pointed out on the basis of $tRNA^{Tyr}$ microsurgery experiments (*12*). Further studies on suppressor tRNAs derived from $tRNA^{fMet}$ and recognized by yeast TyrRS showed the importance of

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase with aa representing Arg, Asp, and Tyr, thus, for arginyl-, aspartyl-, and tyrosyl-tRNA synthetases; tRNA, transfer ribonucleic acid; DTE, dithioerythritol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin.

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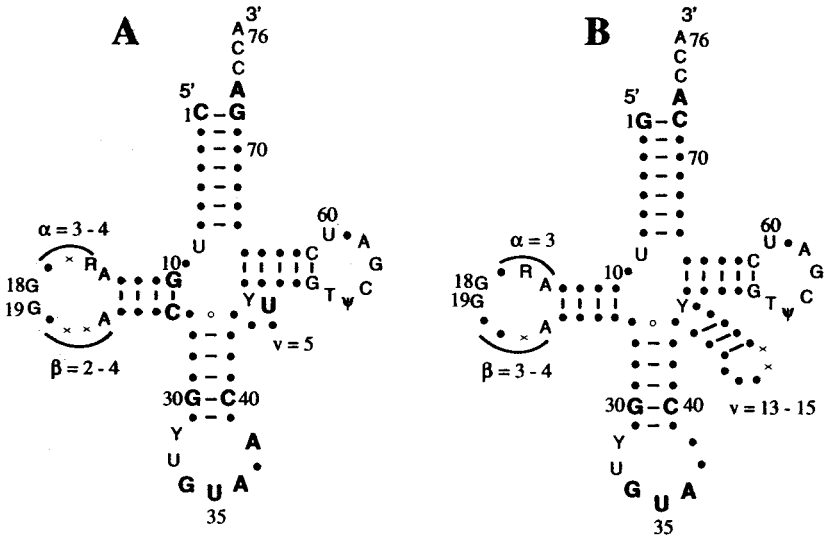

FIGURE 1: Consensus sequences of (A) eukaryotic cytoplasmic $tRNA^{Tyr}$ and (B) eubacterial $tRNA^{Tyr}$. Nucleotides conserved in all tRNAs are given explicitly in standard lettering, whereas nucleotides only specific for $tRNA^{Tyr}$ are in bold. Architectural organization of the D-loop (α- and *β*-domains that define the location of the conserved G18G19 dinucleotide in the loop) as well as the length of the variable region (v) are indicated. Crosses indicate positions that are not conserved. Open circles represent non-Watson−Crick base pairs.

the first base pair C1−G72 and of discriminator residue A73 for recognition by the synthetase (*18*, *19*). This conclusion arose also from studies on minihelix charging by TyrRS from the eukaryotic pathogen *Pneumocystis carinii* (*20*) and is in line with the conservation of the C1−G72 base pair in $tRNA^{Tyr}$ from eukaryotes (Figure 1A). However, no systematic search of the tyrosine identity set for tRNAs recognized by yeast TyrRS has been done to date, and nothing is known about the strength and the interrelation of the tyrosine determinants. It is the aim of this work to fill this gap and to establish this identity set by the in vitro method based on the kinetic analysis of synthetic transcripts derived from the sequence of native $tRNA^{Tyr}$. Data show the dependence of tyrosine identity in yeast on the anticodon loop and especially on the extremity of the amino acid acceptor branch in $tRNA^{Tyr}$. They indicate also that this identity is only marginally dependent on the fine architecture of the tRNA framework in which it is embedded. These conclusions are discussed in the light of the existence of $tRNA^{Tyr}$ mimics.

MATERIALS AND METHODS

Oligonucleotides and Enzymes. Oligonucleotides were from NAPS GmbH (Göttingen, Germany), and L-[^{14}C]-tyrosine (448 mCi/mmol; 1 Ci = 37 GBq) was from Amersham (Les Ulis, France). Restriction enzymes (*Bst*NI, *Hin*dIII, and *Bam*HI) and T4 polynucleotide kinase were from New England Biolabs (Beverly, MA), and T4 DNA ligase was from Boehringer Mannheim (Meylan, France). T7 RNA polymerase was purified from an overproducing strain according to Becker et al. (*21*). The plasmid containing the yeast TyrRS gene was cloned by Chow and RajBhandary (*19*) and overexpressed in BL21(DE3)(pLysS) cells. TyrRS was purified to homogeneity and to a specific activity of 880 units/mg (1 unit charges 1 nmol of tyrosine to tRNA in 1 min in media **1** or **2**, see below) using the same protocol as for yeast ArgRS (*22*).

Preparation of tRNAs. Native yeast $tRNA^{Tyr}$ was purified from enriched counter-current fractions (*23*) by affinity chromatography on a column with immobilized *Thermus thermophilus* EF-Tu-GTP (*24*). Transcripts of $tRNA^{Tyr}$ and its variants were obtained by in vitro transcription of synthetic genes. Since $tRNA^{Tyr}$ gene starts by an unfavorable sequence (5′-CUCUC-3′) for transcription by T7 RNA polymerase, tRNAs were prepared by the "transzyme" method (*2*). It is recalled that this method yields RNA molecules with a 5′-OH group. The $transzyme^{Tyr}$ gene and its mutants correspond to the whole T7 RNA polymerase promoter followed by a hammerhead ribozyme sequence directly connected to the tRNA sequence. A *Bst*NI restriction site coincidental with the 3′-end of the tRNA sequence allows synthesis of molecules terminating with the expected $-CCA_{OH}$ sequence. Purification of the transcripts to single nucleotide resolution was performed by preparative native 12% polyacrylamide gel electrophoresis. The full-length transcripts were eluted from the gel slices using a Schleicher and Schuell Biotrap apparatus (Dassel, Germany). Transplanted yeast $tRNA^{Asp}$ and $tRNA^{Phe}$ transcripts with tyrosine identity determinants, wild-type yeast $tRNA^{Asp}$, and *E. coli* $tRNA^{Tyr}$ with a C1−G72 base pair were synthesized following the same transzyme procedure. Wild-type yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Tyr}$ transcripts were prepared by classical transcription (*25*).

Aminoacylation Reactions. Before aminoacylation, the transcripts were renatured by heating for 2 min at 60 °C, followed by slow cooling to room temperature. Three different buffer conditions were used for aminoacylation. They contained 150 mM Hepes−KOH (pH 7.5), 15 mM $MgCl_2$, and 10 mM ATP (medium **1**), 30 mM Tris-HCl (pH 7.5), 55 mM KCl, 15 mM $MgCl_2$, and 2 mM ATP (medium **2**), and 30 mM Tris-HCl (pH 7.5), 55 mM KCl, 1.1 mM $MgCl_2$, 2 mM ATP, and 1 mM spermine (medium **3**). All media contained 11 μM L-[^{14}C]tyrosine, 0.5 mg/mL BSA, and 20 μM DTE and were complemented by appropriate amounts of tRNA and yeast TyrRS. Some experiments in medium **1** were conducted at pH 6.5 and 8.5 or at pH 7.5 with 0.11 mM L-[^{14}C]tyrosine. After incubation of assay mixtures (100 μL) at 30 °C, aliquots (15 μL) were removed and spotted on Whatman 3 MM paper, and trichloroacetic

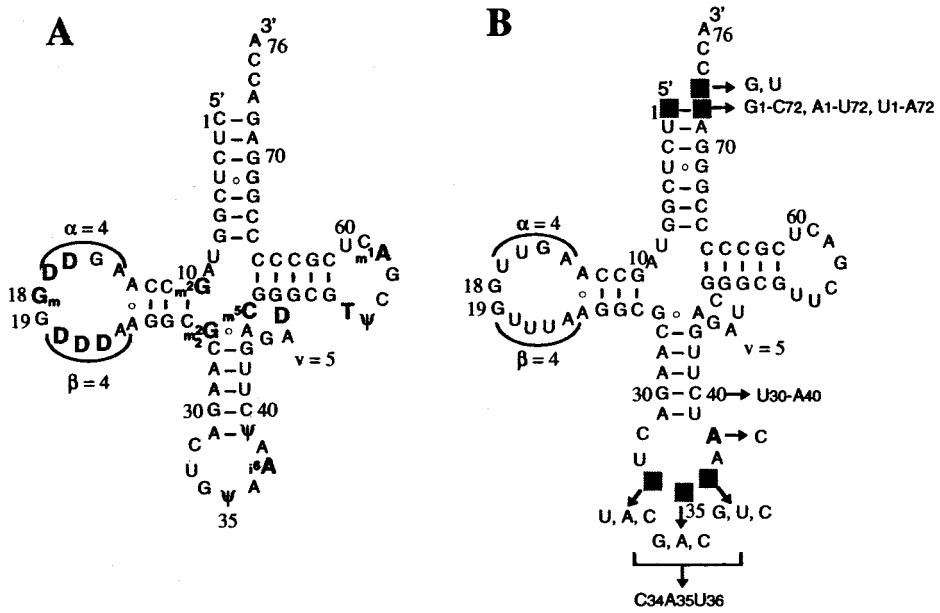

FIGURE 2: (A) Sequences of native yeast $tRNA^{Tyr}$ with modified nucleotides in bold characters and (B) of its unmodified transcript. Mutated positions are in bold characters; arrows indicate the position of mutations and their nature in the different variants. The major determinants for tyrosylation by yeast TyrRS are shaded. The architectural organization of the D-loop, as well as the length of the variable region, are indicated. Open circles represent non-Watson−Crick base pairs.

acid precipitated. Incorporation of radioactive tyrosine was measured by liquid scintillation counting. When not otherwise indicated, aminoacylation assays were conducted in medium **1**. Aminoacylation plateaus were estimated from kinetic curves over 1 h. The apparent kinetic constants (K_M, k_{cat}) were derived from Lineweaver−Burk plots. Displayed data represent an average of at least two independent experiments. Experimental errors on individual kinetic constants are within 15% of the indicated values.

Ultraviolet Melting Measurements. Melting curves for wild-type modified and unmodified $tRNA^{Tyr}$ were recorded on a Uvikon 941 spectrophotometer (Kontron Instruments). Temperature was raised from 15 to 90 °C at a rate of 0.5 °C/min with a NESLAB programmable temperature bath. For measurements, tRNAs were dissolved to a concentration of 0.3 μM in buffers containing either 150 mM sodium cacodylate (pH 7.5), 15 mM $MgCl_2$, or 50 mM sodium cacodylate (pH 7.2) and 10 mM $MgCl_2$. Sodium cacodylate was used because it shows only slight pH variation as a function of temperature.

RESULTS

Aminoacylation and Temperature-Melting of Native $tRNA^{Tyr}$ and Its Unmodified Transcript. The cloverleaf structure of yeast $tRNA^{Tyr}$ with highlighted posttranscriptional modifications is given in Figure 2A. In yeast, $tRNA^{Tyr}$ is among the most modified tRNAs (*13*) with 13 out of its 16 modified residues located in the core of the molecule at positions involved in the network of tertiary interactions responsible for the L-shaped tRNA folding. The sequence of the unmodified transcript is given in Figure 2B. Both molecules are equally well charged by yeast TyrRS to plateau levels of 100%. Kinetic parameters for tyrosylation show slightly enhanced K_M and k_{cat} values for the native molecule which results in a ~2-fold loss of aminoacylation efficiency (as defined by the k_{cat}/K_M ratio) for the unmodified transcript (Table 1).

Table 1: Kinetic Parameters for Tyrosylation by Yeast TyrRS of Yeast $tRNA^{Tyr}$ and Variants Derived from $tRNA^{Tyr}$ Transcript

tRNAs	plateau (%)	k_{cat} ($\times 10^{-3}$ s^{-1})	K_M (μM)	L (x-fold)[a]
wild-type molecules				
native tRNA	100	1500	2.0	0.6
unmodified transcript	100	440	1.0	1
acceptor stem variants				
C1-G72 → U1-A72	2.5	1.1	20	8000
C1-G72 → A1-U72	0.5	0.3	12	18 000
C1-G72 → G1-C72	<0.2	nm[b]	nm	nm
A73 → U73	2.5	0.55	10	8400
A73 → G73	2.5	0.35	15	19 000
anticodon stem and loop variants				
G34 → U34	13	5.9	6.2	470
G34 → C34	10	3.2	7.3	1000
G34 → A34	6	1.0	3.3	1500
U35 → A35	43	3.8	1.2	140
U35 → C35	40	6.1	2.4	170
U35 → G35	40	3.0	1.3	190
A36 → G36	39	10	4.6	200
A36 → C36	40	10	5.0	220
A36 → U36	35	8.5	4.6	230
A38 → C38	100	240	1.3	2.4
G30-C40 → U30-A40	100	420	1.8	1.8
G34U35A36 → C34A35U36	2	0.3	8.1	11 000

[a] L values are the inverse of the aminoacylation efficiency relative to the wild-type transcript, taken as reference. Relative aminoacylation efficiencies are defined as $(k_{cat}/K_M)_{relative} = (k_{cat}/K_M)_{mutant}/(k_{cat}/K_M)_{wild-type}$. [b] Not measurable.

Comparative thermal melting profiles of native and unmodified $tRNA^{Tyr}$ are displayed in Figure 3. In solutions mimicking an aminoacylation medium, the native molecule is significantly stabilized as compared to the unmodified transcript (T_m = 72.5 versus 67 °C). Noticeably, the melting is much more cooperative for the native molecule. Melting temperatures are dependent on the nature of the buffer. In 50 mM sodium cacodylate (pH 7.2) and 10 mM $MgCl_2$, they are 77 and 70 °C for the native and unmodified $tRNA^{Tyr}$ (data not shown) compared to the 72.5 and 67 °C in 150

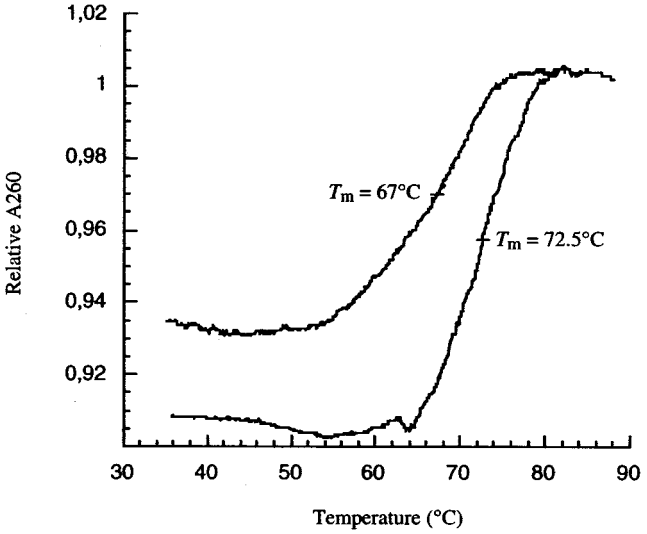

FIGURE 3: Normalized ultraviolet absorbance melting curves of modified (bottom curve) and unmodified (top curve) wild-type $tRNA^{Tyr}$ in 150 mM sodium cacodylate (pH 7.5) and 15 mM $MgCl_2$. Absorbance was monitored at 260 nm.

mM sodium cacodylate (pH 7.5) and 15 mM $MgCl_2$ (Figure 3).

Tyrosylation of $tRNA^{Tyr}$ Variants. Seventeen variants were tested for tyrosylation (Figure 2B). The positions of isolated nucleotides or base pairs, expected to contribute in a major way to tyrosine identity, were mutated in their three possible versions (except mutant C73 at the discriminator position that could not be transcribed for unknown reasons despite the correct sequence of the variant tDNA gene). Functional data are listed in Table 1. All three variants of base pair C1−G72 exhibit very poor charging levels, reflecting a weak activity of these molecules. Their K_M is enhanced 10−20-fold and is even not measurable for the G1−C72 variant, and their k_{cat} is reduced ∼1000-fold. As compared to the transcript with wild-type sequence, the loss in aminoacylation efficiency is of ∼4 orders of magnitude. A similar kinetic behavior is observed when the discriminator base A73 is mutated into G73 or U73. In the anticodon loop, the strongest effects occur for mutations at position 34, with losses in efficiency varying from 470 to 1500 according to the nature of the mutated residue. Variants with mutations at positions 35 and 36 are almost insensitive to the nature of the mutated residue. For both positions, the drop in efficiency of aminoacylation is between 140 and 230-fold, mainly due to a lowering of k_{cat}. The triple mutation changing the tyrosine anticodon GUA in the methionine anticodon CAU induces a dramatic loss of aminoacylation efficiency (∼11000-fold), essentially due to a decreased k_{cat}. The other mutants tested (at positions 38 and 30−40) have only a marginal effect on tyrosylation.

Tyrosylation under Different Aminoacylation Conditions. Tyrosylation of native $tRNA^{Tyr}$ and of selected transcripts was investigated in three incubation media, differing by the nature and the concentration of buffer and the concentration of KCl, ATP, $MgCl_2$, and/or spermine (Table 2). While the charging of native $tRNA^{Tyr}$ is almost insensitive to incubation conditions, that of $transcript^{Tyr}$ and its variants varies significantly. For $transcript^{Tyr}$, losses in aminoacylation efficiency range from 0.4 to 8 and are most affected in medium **3**. Similarly, behavior of the variants is strongly dependent on the composition of the incubation medium. These variations are not due to different amounts of active molecules in the different aminoacylation media, since the tyrosylation plateau for each of these molecules is insensitive to the nature of the incubation medium (data not shown). Tyrosylation of the variants is mainly affected at the k_{cat} level, whereas K_M is globally unchanged, except for variant A34 in medium **2** and variant U36 in medium **3**.

Tyrosylation characteristics of wild-type $tRNA^{Tyr}$ transcript were also determined in medium **1** at pH 6.5 and 8.5 for both wild-type transcript and anticodon variant G35. Furthermore, charging of the wild-type transcript and of two

Table 2: Influence of Assay Conditions on Kinetic Parameters for Tyrosylation of Wild-Type and Variant $tRNA^{Tyr}$ Molecules

Variable Elements in Tyrosylation Media

	medium **1**[a]	medium **2**[b]	medium **3**[c]
Tris-HCl, pH 7.5 (mM)	0	30	30
hepes−KOH, pH 7.5 (mM)	150	0	0
KCl (mM)	0	55	5
$MgCl_2$ (mM)	15	15	1.1
ATP (mM)	10	2	2
spermine (mM)	0	0	1

Kinetic Parameters for tRNA Tyrosylation

tRNAs	k_{cat} ($\times 10^{-3}$ s^{-1})	K_M (μM)	k_{cat} ($\times 10^{-3}$ s^{-1})	K_M (μM)	k_{cat} ($\times 10^{-3}$ s^{-1})	K_M (μM)	range of *L* values[e]
wild-type molecules							
native $tRNA^{Tyr}$	1500	2.0	2700	1.0	2800	2.0	0.3−0.6
wild-type transcript	440	1.0	1300	1.1	57	1.0	0.4−8
anticodon variants							
G34 → A34	1.0	3.3	9.7	10	0.6	7.5	450−5500
U35 → C35	6.1	2.4	85	3.1	2.0	2.8	16−176
U35 → G35	3.0	1.3	13	3.0	nd[d]	nd	102−619
A36 → U36	8.5	4.6	39	3.9	4.3	15	44−1571
range of *L* values[f]	0.6 − 1500		0.2−450		0.3−5500		

[a] Medium **1** corresponds to standard conditions used in this work. [b]Medium **2** corresponds to standard conditions used in ref *12*. [c]Medium **3** was also used in ref *12* and is derived from conditions that should increase aminoacylation specificity (*39*). [d]Not determined. *L* values are the inverse of the kinetic efficiency relative to the wild-type transcript in medium **1**, taken as reference. [e]For a given tRNA tested in the three media, [f]for the different tyrosine accepting molecules tested in a given medium.

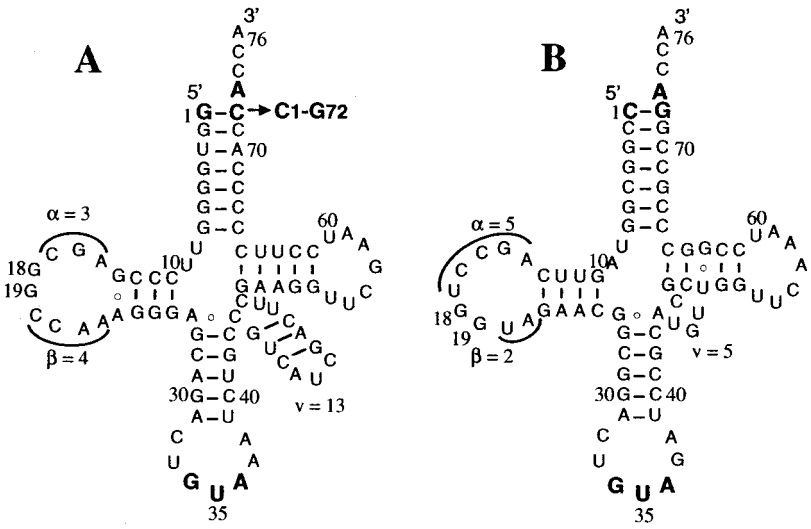

FIGURE 4: Cloverleaf structures of (A) eubacterial (*E. coli*) and (B) archaeal (*M. jannaschii*) $tRNA^{Tyr}$ with yeast tyrosine identity nucleotides in bold characters. The first base pair G1−C72 of *E. coli* $tRNA^{Tyr}$ was mutated into C1−G72 (shown by arrows). The ribonucleotide sequence for eubacterial $tRNA^{Tyr}$ is from Goodman et al. (*52*), and that for archaeal $tRNA^{Tyr}$ is derived from the genome (*53*).

Table 3: Kinetic Parameters for Tyrosylation by Yeast TyrRS of Noncognate tRNAs and of Transplanted tRNAs

tRNAs	plateau (%)	k_{cat} ($\times 10^{-3}$ s^{-1})	K_M (μM)	L (x-fold)[a]
yeast $tRNA^{Tyr}$	100	440	1.0	1
M. jannaschii $tRNA^{Tyr}$	100	100	2.4	10
E. coli $tRNA^{Tyr}$	<1	nm[b]	nm	nm
$tRNA^{Asp}$	<0.1	nm	nm	nm
$tRNA^{Phe}$	<0.1	nm	nm	nm
E. coli $tRNA^{Tyr\,(C1-G72)}$	100	660	1.3	0.9
yeast $tRNA^{Asp\rightarrow Tyr}$	100	530	0.6	0.5
yeast $tRNA^{Phe\rightarrow Tyr}$	100	130	1.5	5

[a] L values are the inverse of the aminoacylation efficiency relative to the wild-type transcript, taken as reference. [b] Not measurable.

variants, U34 and A1-U72, was analyzed in the presence of a 10-fold increased tyrosine concentration. Under all these conditions, no notable differences with the values of kinetic parameters determined at pH 7.5 under standard conditions (11 μM tyrosine) were observed (data not shown).

Tyrosylation of Heterologous $tRNA^{Tyr}$ Species. Sequence of heterologous tyrosine-specific tRNA transcripts from *E. coli* and *M. jannaschii* prepared by in vitro transcription is displayed in Figure 4 and their tyrosylation characteristics shown in Table 3. Activity of *M. jannaschii* $tRNA^{Tyr}$ is close to that of the homologous yeast transcript (only a 10-fold loss in aminoacylation efficiency). In contrast, the transcript of *E. coli* $tRNA^{Tyr}$ is completely inactive. Interestingly, replacing base pair G1−C72 by C1−G72, as in yeast $tRNA^{Tyr}$, yields a fully active molecule with kinetic parameters equivalent to those of the yeast tRNA (Table 3).

Identity Swaps. Molecules based on the sequence of yeast $tRNA^{Asp}$ and $tRNA^{Phe}$ and containing the six putative identity elements were created (Figure 5). Since two such elements are already present in $tRNA^{Asp}$, only four nucleotides were changed in its sequence. In the case of yeast $tRNA^{Phe}$ possessing three tyrosine elements, transplantation concerned three residues. Plateaus and kinetic parameters for tyrosylation of these molecules are almost as for wild-type $tRNA^{Tyr}$ transcript (Table 3). Only faint differences were found; the largest concerns $tRNA^{Phe\rightarrow Tyr}$, which is a 5-fold less efficient substrate for TyrRS than $tRNA^{Tyr}$, due to a decreased k_{cat} (Table 3). Control experiments indicate that the nontransplanted $tRNA^{Asp}$ and $tRNA^{Phe}$ molecules are not substrates of TyrRS.

Tyrosylation of $tRNA^{Tyr}$ with 5′- and 3′-Shifted Anticodons. Two $tRNA^{Tyr}$ variants with their anticodon triplet GUA shifted by one nucleotide, either to the 5′- or to the 3′-side, were assayed (Figure 6). The molecule with the anticodon 3′-shifted (G35U36A37) is charged to an appreciate plateau level (40%) and has an aminoacylation efficiency only reduced 280-fold as compared to the wild-type transcript, mainly due to a decreased k_{cat}. In contrast, the molecule with the 5′-shift (G33U34A35) is only charged to a plateau level of 2% and has its aminoacylation efficiency decreased by ~4 orders of magnitude.

DISCUSSION

Nonimportance of Modified Nucleotides and of 5′-Phosphate for Tyrosylation. Modified bases have been shown so far to act as identity elements in only isoleucine, lysine, and glutamine systems from *E. coli* (*26*−*30*). Due to the large number of posttranscriptional modifications in yeast $tRNA^{Tyr}$ (*31*) and more generally in $tRNA^{Tyr}$ species from many organisms (*13*), one could wonder about their possible implication in tyrosylation. Our data show that the posttranscriptional modifications do not play a direct role in the tyrosine identity in yeast (although antideterminant effects cannot be excluded). In particular, Ψ35, at an identity position in the anticodon, does not participate in the identity since the unmodified transcript is fully active.

It should also be noticed that most investigated transcripts were lacking the 5′-phosphoryl group on their first nucleotides. Since the wild-type transcript is fully active, this means that this phosphoryl group is unimportant for tyrosylation despite the importance of the C1−G72 base pair.

Conformation of Modified and Unmodified $tRNA^{Tyr}$. Temperature-melting profiles of the native $tRNA^{Tyr}$ and its unmodified transcript indicate significant differences in the structural stability of the two molecules. The modified tRNA melts at a temperature ~5 °C higher than the transcript and in a much more cooperative fashion. The absolute values of

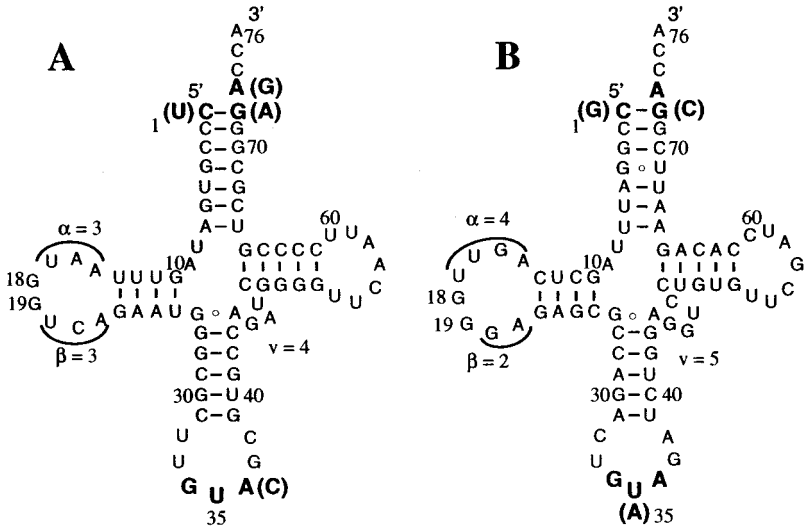

FIGURE 5: Cloverleaf structures of transplanted (A) yeast $tRNA^{Asp}$ (*54*) and (B) yeast $tRNA^{Phe}$ (*55*). Yeast tyrosine identity determinants are in bold characters, and the mutated nucleotides are given in brackets. Architectural organization of the D-loop (α- and *β*-domains that define the location of the conserved G18G19 dinucleotide in the loop) as well as the length of the variable region (v) are indicated. Open circles indicate non-Watson−Crick base pairs.

	a	b	c	d
tRNAs	$tRNA^{Tyr}$	$tRNA^{Tyr}$	$tRNA^{Tyr}$	BMV RNA
plateau (%)	100	2	40	40 (*)
k_{cat} ($s^{-1}x10^3$)	440	0.5	6.5	(**)
K_M (μM)	1.0	16	4.2	(**)
Loss (x-fold)	1	14000	280	

FIGURE 6: Sequence of the anticodon loop region of tyrosine accepting RNAs and kinetic parameters for tyrosylation by yeast TyrRS. (a) Data for wild-type yeast transcript^{Tyr}, (b) for transcript^{Tyr} with 5′-shifted anticodon, (c) for transcript^{Tyr} with 3′-shifted anticodon, and (d) for Brome Mosaic Virus (BMV) tRNA-like domain, with loop B3 as defined by Felden et al. (*5*). The tyrosine anticodon sequence is in bold characters. Losses in aminoacylation efficiency (*L*) are defined relative to the wild-type $tRNA^{Tyr}$ transcript taken as the reference. (*) Data from Dreher and Hall (*45*) with wheat germ TyrRS; (**) the maximal velocity of tyrosylation was found 200-fold lower than for $tRNA^{Tyr}$, but the K_M suggests that the viral RNA has a better affinity than cognate tRNA for TyrRS (*38*).

melting temperatures are dependent upon experimental conditions, in particular on magnesium ion concentration. This behavior is reminiscent with that observed for a set of couples of modified and unmodified tRNAs where the native molecule is always the most stable (e.g., refs *32* and *33*).

Determinants in tRNA for Yeast TyrRS. The consensus sequence of eukaryotic cytoplasmic $tRNA^{Tyr}$ reveals 12 conserved residues (Figure 1A). Those which belong often to tRNA identity sets (*1*), as the three anticodon residues and the discriminator base, and those unique to yeast $tRNA^{Tyr}$, as the first base pair C1−G72, were mutated three times each. Residues less often involved in identity were either mutated once (U30−A40 pair, A38) or were checked in transplanted tRNAs. This concerned position 47 and base pair 10−25, which are A47 and G10-U25 in yeast $tRNA^{Asp}$. We took also into consideration the algorithm of Nicholas and McClain (*34*), which points out 22 nucleotides being the most characteristic for $tRNA^{Tyr}$ in yeast (with disjointedness counts greater than 25). These 22 nucleotides were tested individually or in groups for their effect on tyrosylation. This was done either directly in appropriate mutated $tRNA^{Tyr}$ transcripts or indirectly in nontRNA^{Tyr} molecules in which some of them were transplanted.

From the study of the mutated $tRNA^{Tyr}$ transcripts, it appears that the tyrosine identity set includes six nucleotides. They are the three anticodon residues (G34, U35, and A36) and three residues in the acceptor stem (base pair C1−G72 and discriminator residue A73). The contribution of the nucleotides in the acceptor stem is by far the most important as activity losses due to their mutation exceed at least by 1 order of magnitude those due to mutation of the anticodon identity residues. The strength of anticodon determinants, however, is much more pronounced as shown previously (*12*). This discrepancy probably is only apparent and reflects experimental differences in the two studies: tyrosylation measurements conducted on unmodified transcripts in the present study and on variants with modifications in (*12*) and differences in the TyrRS preparations used.

Expression of identity sets has been shown in several cases to be context and structure dependent (e.g., refs *35*−*37*). Therefore, the integrity of the tyrosine set was tested in four different sequence and structure contexts. First, it was tested in *M. jannaschii* $tRNA^{Tyr}$, which contains the six tyrosine elements embedded in a sequence only marginally different from that of the yeast tRNA. Second, it was tested in yeast $tRNA^{Asp}$ and $tRNA^{Phe}$, which have a small variable region of four to five residues but show large structural differences with $tRNA^{Tyr}$ in size and organization of the D-loop. Third, it was tested in *E. coli* $tRNA^{Tyr}$ that contains an incomplete yeast tyrosine identity set but presents a large variable region. All these molecules, either native or transplanted, are fully charged by yeast TyrRS with kinetic parameters close to those of the cognate $tRNA^{Tyr}$.

Altogether, the activity of the transplanted tRNAs confirms that the six residues retained by the mutational analysis (base pair C1−G72 and residues G34, U35, A36, and A73)

constitute the complete tyrosine identity set and that its expression is only marginally sensitive to, if any, sequence or architectural features of the tRNA. In particular, the presence of a large variable region does not influence tyrosylation.

In retrospect, it is worth noticing that the yeast tyrosine identity set is included within the 12 conserved residues in eukaryotic $tRNA^{Tyr}$ species. This agrees with the general view that identities are essentially conserved during evolution, in particular within the great kingdoms in the evolutionary tree of life (*1*). Interestingly, however, two out of the six identity nucleotides are not included within the set of 22 nucleotides that distinguish best $tRNA^{Tyr}$ from other yeast tRNAs (*34*). Those are the discriminator A73 and anticodon residue G34. This fact points to another general feature in tRNA identity, namely the importance of the anticodon and discriminator positions (reviewed in ref *1*). Although, individual residues at these positions cannot determine alone a tRNA identity, they can contribute strongly to this identity by acting in synergy.

Factors Modulating Expression of Tyrosine Identity. Previous investigations on tRNA charging by yeast TyrRS do not allow easy comparison of results. For instance, a K_M of 0.5 μM for the native tRNA was reported in one study (*38*) and of 0.05 μM in another one (*12*). Moreover, tyrosylation efficiency (k_{cat}/K_M) of native $tRNA^{Tyr}$ and of variants with mutations at positions 34 and 35 was shown to be strongly dependent on assay conditions, essentially as the result of K_M variations (*12*), in contrast with this study, where it is mainly due to k_{cat} variations.

To clarify these points, we tested a series of tRNAs and searched for variations of tyrosylation efficiency caused by differences in the composition of the aminoacylation media. The three conditions tested were adapted from those described in the above-mentioned papers. They differ in ATP and $MgCl_2$ concentrations and by the replacement of part of $MgCl_2$ by spermine [a compound known to increase the specificity of tRNA recognition by synthetases (*39*)]. In assays conducted in parallel with the same pure TyrRS preparation, no significant differences were found when comparing the activity of native (i.e., modified) $tRNA^{Tyr}$ in the three media (Table 2). The deduced K_M values (1−2 μM) were in the same range as reported previously (*38*). This contrasts with the condition-dependent data obtained with a partially purified enzyme (*12*). A sensitivity to reaction conditions was found here with the unmodified $tRNA^{Tyr}$ and with the variants. This sensitivity may reflect a more flexible conformation of the $tRNA^{Tyr}$ transcript as suggested by the melting measurements. For the wild-type transcript, the k_{cat} for tyrosylation varied indeed by a factor of 20, being highest in medium **1** (1.3 s^{-1}) and lowest in medium **3** (0.057 s^{-1}), while the K_M remained unaffected. As to the mutants, variations were found for both K_M and k_{cat}. In particular, for molecules modified at positions 34 and 35, the amplitude (but not the absolute value) of the K_M effect is comparable to that observed by Bare and Uhlenbeck (*12*). In addition, in medium **1**, it was found that changing the pH from 6.5 to 8.5 did not influence the efficiency of tyrosylation of the wild-type transcript and of an anticodon variant.

In conclusion, the comparative functional assays emphasize a great sensitivity of the tyrosylation reaction to external factors. This sensitivity is dependent upon the tRNA and is most pronounced with the transcripts. It is not related to tRNA-dependent binding effects of free tyrosine to TyrRS that could lead to subsaturating amino acid concentration with certain tRNA variants and thus influence their aminoacylation process, as evidenced in the glutaminyl and tryptophanyl systems (*40*). Indeed, no such tRNA dependence was observed when comparing tyrosylation parameters determined at 0.11 mM or 11 μM tyrosine. We propose therefore that the observed kinetic effects are likely due to faint condition-dependent structural changes in the tRNA transcript structures. The fact that strongest differential effects are observed in medium **3**, at conditions favoring specificity (*39*), gives support to this interpretation. From a practical point of view, this means that informative comparisons of aminoacylation activities of tRNA variants can only originate from measurements conducted under strictly similar conditions.

Structural and Functional Peculiarities of the Yeast Tyrosine System. It is a common trend that expression of identities is different in systems involving class I and class II synthetases. While in class I systems, the strongest identity elements are mostly found in the anticodon loop, they are often comprised in the acceptor stem in class II systems (*1*). The tyrosine systems, with a class I synthetase, is a case that deviates from this scheme, since experimental data are better interpreted in the frame of a class II system. This assumption is based on the following arguments. First, the tyrosine identity nucleotides in the acceptor stem of $tRNA^{Tyr}$ have by far the strongest effect on tyrosylation. Second, when the major determinant C1−G72 is mutated into G1−C72, tyrosylation of the variant is completely abolished, despite the two base pairs having similar functional groups in the minor groove (*41*), the side of the tRNA is believed to be recognized by class I synthetases (*42*). Thus, inactivity of the G1−C72 mutant implies that TyrRS recognizes the major instead of the minor groove side of the acceptor stem. Similar mutational studies of the first base pair in a suppressor of $tRNA^{fMet}$ recognized by TyrRS (*18*) and in $microhelix^{Tyr}$ from *P. carinii* (*20*) lead to the same conclusion. Likewise, in the tyrosine system from *Bacillus stearothermophilus*, a model of the tRNA-synthetase complex based on a mutational analysis of the synthetase, implies approach of the tRNA by TyrRS from the major groove side of the acceptor stem (*43*). Third, the triple mutant of $tRNA^{Tyr}$ anticodon (GUA → CAU) shows rather high activity (see Table 1) and behaves as a triple anticodon mutant of $tRNA^{Asp}$ aminoacylated by class II AspRS (*44*). While in case of an additive effect, the triple mutant should have a loss of efficiency of $\sim 10^7$, it is only 11000-fold less active than wild-type $tRNA^{Tyr}$, which suggests that identity is mainly dictated by the determinants in the acceptor stem.

Anticodon Recognition by Yeast TyrRS. Yeast TyrRS charges the 3′-terminus of the RNA from Brome Mosaic Virus (*38*, *45*, *46*). This suggests the presence of the tyrosine identity elements in the tRNA-like structure recognized by TyrRS. Surprisingly, no equivalent of a seven-residue anticodon loop with a tyrosine anticodon exists in the viral RNA-domain that mimics $tRNA^{Tyr}$ (*5*). Instead one finds a tetraloop (Figure 6d) closing a helical stem mimicking the anticodon branch of $tRNA^{Tyr}$. Although this tetraloop is in proximity with TyrRS (*38*), one can question about its involvement in tyrosine identity, unless one supposes that

an A from the tetraloop substitutes for the identity determinant A36 present in $tRNA^{Tyr}$. On the other hand, it was shown that the identity nucleotides in the anticodon loop of arginine accepting tRNAs can be recognized by yeast ArgRS in alternate locations within this loop. While in $tRNA^{Arg}$, C35 and G36 are identity determinants, they are 3′-shifted to positions 36 and 37 in the $tRNA^{Asp}$ transcript, which is an efficient arginine acceptor (*47*, *48*). Such phenomenon may also occur with TyrRS, a class I synthetase like ArgRS. To shed light on the exact role of the anticodon in tyrosine identity, we have investigated $tRNA^{Tyr}$ mutants with 5′- and 3′-shifted anticodons.

The tRNA with the 5′-shifted anticodon is of particular interest. As the viral tRNA-like domain, it has a disturbed anticodon loop with conserved U33 replaced by G33. In the case of a nonimportance of the anticodon loop conformation, this molecule may retain strong tyrosylation activity. Experiments do not confirm this expectation since the tRNA with the 5′-shifted anticodon is poorly charged to a plateau of only 2% and with an efficiency dropped 14000-fold. The situation is different for the variant with the 3′-shifted anticodon, which keeps a canonical anticodon loop with U33 and A37 like wild-type $tRNA^{Tyr}$. This molecule is in fact a strict double mutant at positions 35 and 36, which in the case of additivity of the mutations should have its activity reduced ~44000-fold (i.e., 190 × 230). Experiments show a decrease of tyrosylation efficiency by only 280-fold (i.e., ~150-fold less), indicating that this molecule either behaves as the tRNA-like structure or triggers strong compensatory effects leading to a different recognition mechanism of the shifted GUA anticodon by TyrRS. This second interpretation is in line with what is observed for tRNA recognition by class I ArgRS, which is context dependent and is ensured by alternate recognition sets in the anticodon loop of the accepting tRNAs (*47*).

Concluding Remarks. This work brings further support to the general concept explaining specificity of tRNA charging by synthetases. To achieve their function, synthetases must recognize identity nucleotides properly located in appropriate structural RNA frameworks. In the case of the yeast tyrosine system, this framework can tolerate substantial structural alteration, as those found in tRNA mimics (*49*), including the intricate structure of the tRNA-like domain in Brome Mosaic Virus RNA (*5*). Altogether, this view is in line with the structural diversity that exists in both aminoacylatable RNAs and in synthetases (*49*−*51*).

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