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Use of RNase P for Efficient Preparation of Yeast tRNA^{Tyr} Transcript and Its Mutants

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Because T7 RNA polymerase has a strong preference for particular sequences to initiate transcription, some RNAs having pyrimidine-rich sequences at their 5′-end (yeast tRNA^{Tyr}, for example) are hardly transcribed by this enzyme. To circumvent this inconvenience, we have developed an efficient method for *in vitro* preparation of such tRNAs. The RNA of interest is first transcribed as a precursor form that has purine-rich extra sequences at its 5′-end, then processed with RNase P to generate the objective tRNAs. By using this protocol, we were able to prepare easily and efficiently yeast tRNA^{Tyr} transcript and its mutants harboring base substitutions within the anticodon loop and/or acceptor stem regions. Aminoacylation analyses of these tRNA transcripts with yeast tyrosyl-tRNA synthetase revealed that the replacement of G34 by C34 (mutation to amber suppressor) severely impaired the aminoacylation, whereas the replacement of the U4:G69 wobble base-pair in the acceptor stem region by C4:G69 normal Watson-Crick type base-pair improved it.

Key words: aminoacylation, *in vitro* transcription, RNase P, transfer tRNA, tyrosyl-tRNA synthetase.

Abbreviations: aaRS, aminoacyl-tRNA synthetase; IPTG, isopropyl-thio-β-D-galactoside; TyrRS, tyrosyl-tRNA synthetase [EC 6.1.1.1].

During attempts to utilize yeast tRNA^{Tyr}/TyrRS pair as the "carrier" of non-canonical (unnatural) amino acid(s) in an *Escherichia coli* translation system, we have developed an efficient system for co-expression of yeast amber suppressor tRNA^{Tyr} (anticodon; CUA) and wild-type TyrRS in *E. coli* cells. The expressed yeast suppressor tRNA^{Tyr} was shown to be charged with tyrosine only by its cognate yeast TyrRS and not by *E. coli* TyrRS or other aminoacyl-tRNA synthetases (aaRS) (*1*). We have also succeeded in constructing a mutant of yeast TyrRS that can aminoacylate tRNA^{Tyr} (or its suppressor derivative) with 3-substituted tyrosine analogues instead of L-tyrosine (*2*). Thus, this extra tRNA/TyrRS pair seemed to be a possible bridgehead for developing the system for site-directed incorporation of unnatural amino acids into proteins. However, at least two papers published since then have reported that yeast amber suppressor tRNA^{Tyr} could be slightly aminoacylated by *E. coli* endogenous aaRS(s) in some circumstances (*3*, *4*). We ourselves also detected a trace amount of misaminoacylation depending on the balance of tRNA/aaRS levels when this tRNA was used in an *in vitro* protein-synthesizing system. Such misaminoacylation could cause a "double-meaning" in the amino acid assignment for the amber UAG codon and eventually deteriorate the homogeneity of product proteins in which unnatural amino acid is incorporated at the amber-mutated site.

Although it is desirable to optimize the nucleotide sequence of yeast amber suppressor tRNA^{Tyr} so that it is not recognized by aaRSs other than yeast TyrRS, there is an intrinsic difficulty in preparing mutant sequences of yeast tRNA^{Tyr}. Since this tRNA has a pyrimidine-rich tract CUCUC at the 5′-terminus, the usual *in vitro* transcription method using T7 RNA polymerase cannot be adopted for preparation of such a tRNA or related RNAs. To make matters worse, C1 at the 5′-end cannot be replaced by purine nucleotides because the first base-pair C1:G72 is the major identity element for eukaryotic tRNA^{Tyr} (*5*). In order to circumvent this inconvenience, we have developed an efficient *in vitro* method for preparing a series of mutant yeast tRNA^{Tyr}s by the use of *E. coli* RNase P (*6*, *7*). We report here the protocol for preparing yeast tRNA^{Tyr} transcript and its mutants, including amber suppressor tRNA^{Tyr} and mutants with U4 to C4 substitution, and the results of aminoacylation analyses of these tRNA transcripts.

EXPERIMENTAL PROCEDURES

General—Restriction enzymes were obtained from Fermentas, and *Taq* DNA polymerase and Klenow fragment from Takara Shuzo. Plasmids pUC19, pET21a (+) and pGEMEX-1 were obtained from Takara Shuzo, Novagen and Promega, respectively. Yeast TyrRS and native tRNA^{Tyr} were prepared as described (*8*). SP-Sepharose high performance and Q-Sepharose high performance are products of Amersham Biosciences. L-[^{14}C]-Tyrosine (14.65 GBq/mmol) was obtained from Amersham Biosciences. Other chemicals were obtained from Wako Pure Chemicals, Japan.

Preparation of the Protein Component (C5 Protein) of RNase P—The gene (*rnpA*) for C5 protein (*6*) was

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PCR-amplified from *E. coli* JM109 genomic DNA. The primers used were 5′-GGG GCT GCA GCA TAT GGT TAA GCT CGC ATT TCC CAG-3′ and 5′-GGG GCT CGA GCC TGG GCG CTC GGT CCG CTG-3′ to incorporate *Nde*I and *Xho*I sites (underlined), respectively. The PCR fragment was then cloned into plasmid pET21a (+) to create pETC5. *E. coli* BL21(DE3) cells containing pETC5 were grown to an OD_{600} of 0.5–0.8 in 1 liter of Luria-Bertani medium and induced with 0.5 mM IPTG. After 4 h of cultivation at 37°C, expressed cells were harvested and stocked frozen. Frozen cells were re-suspended in 50 ml of 50 mM Tris-HCl (pH 7.6), 5 mM EDTA, 10% glycerol and 1 M NaCl, and disrupted by a sonicator (Bioruptor, Tohsho Denki, Japan). After centrifugation at 30,000 × *g* for 30 min, the supernatant was treated with 50% saturated ammonium sulfate. After centrifugation at 17,000 × *g* for 15 min, the supernatant was precipitated with 80% saturated ammonium sulfate. The precipitate was recovered by centrifugation at 17,000 × *g* for 15 min and dissolved in buffer A [50 mM sodium acetate (pH 6.5), 5 mM EDTA and 0.25 M NaCl], and the solution was dialyzed against buffer A. The dialyzed protein solution was applied onto a 10-ml SP-Sepharose high performance column. The column was developed with a linear gradient of NaCl (0.25–1.0 M, total 200 ml) at a flow rate of 2.0 ml/min. Fractions containing C5 protein were collected, concentrated by Amicon Ultra, dialyzed against 20 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 40 mM KCl, 6 mM 2-mercaptoethanol and 50% glycerol at 4°C overnight, and stored at –30°C.

Preparation of the RNA Component (M1 RNA) of RNase P—The gene (*rnpB*) for M1 RNA (*7*) was PCR-amplified from *E. coli* JM109 genomic DNA. The primers used were 5′-GGG GGA ATT CTA ATA CGA CTC ACT ATA GAA GCT GAC CAG ACA GTC GC-3′ to fuse an *Eco*RI site and T7 promoter (underlined) and 5′-GGG GGG ATC CGG ATG ACG TAA ATC AGG TGA AAC TG-3′ to incorporate a *Bam*HI site (underlined). The PCR fragment was then cloned into plasmid pUC19 to create pUCM1. The template for *in vitro* transcription of M1 RNA was prepared by PCR-amplification from pUCM1 with a T7 primer hybridized to the T7 promoter region (5′-GGG GCT GCA GTA ATA CGA CTC ACT ATA-3′) and a primer which is complementary to the 3′ terminal region of M1 RNA (5′-AGG TGA AAC TGA CCG ATA AG-3′). *In vitro* transcription was performed according to the method of Sampson and Uhlenbeck (*9*). After transcription, the reaction mixture was applied onto a column (1 ml) of Q-Sepharose high performance. The column was developed with a linear gradient of NaCl (0.4–1.0 M, total 20 ml) at a flow rate of 0.5 ml/min. M1 RNA was recovered by ethanol precipitation from the eluate.

Construction of Plasmids Harboring Wild-Type or Mutant $tRNA^{Tyr}$ Genes—The plasmid designated pGEMEX-supTyr for amber suppressor $tRNA^{Tyr}$ was constructed as follows. Two oligodeoxynucleotides containing 17-nucleotide-long complementary regions (underlined), 5′-GGG GTC TAG ACT CTC GGT AGC CAA GTT GGT TTA AGG CGC AAG ACT CTA AAT CTT GAG ATC GGG C-3′ and 5′-GGG GAA GCT TGG TCT CCC GGG GGC GAG TCG AAC GCC CGA TCT CAA GAT TT-3′, were annealed and converted to double-stranded DNA with Klenow fragment by incubation at 37°C for 1 h. The synthetic amber suppressor tRNA gene was digested with *Xba*I and *Hin*dIII, and then ligated to the *Xba*I and *Hin*dIII sites of pGEMEX-1. The sequence of the amber suppressor tRNA gene was confirmed by the dideoxy sequencing according to a manufacturer's protocol (Amersham). The other plasmids containing the genes for wild-type yeast $tRNA^{Tyr}$ or mutant tRNAs harboring a base substitution within the acceptor stem region were generated similarly. Each mutation was confirmed by the dideoxy sequencing.

Transcription and In Vitro *Processing of tRNA*—As mentioned above, yeast $tRNA^{Tyr}$ has a 5′ terminal sequence unfavorable to transcription by T7 RNA polymerase. Therefore, we first transcribed yeast $tRNA^{Tyr}$ or its mutants as a precursor (pre-tRNA) form that has 27 extra nucleotides including 5′-GGGAGA-3′ (the sequence preferred by T7 RNA polymerase) at the 5′ end, then processed them with RNase P to generate mature tRNAs. The overall strategy is schematically shown in Fig. 1. The template for *in vitro* transcription of pre-tRNA was prepared by PCR-amplification of a pGEMEX-1 plasmid harboring the suitable tRNA gene by using a T7 primer (5′-AAT TGC TGC AGT AAT ACG ACT CAC TAT A-3′) and an OMe primer [5′-T(Gm)G TCT CCC GGG GGC GAG T-3′], which is complementary to the 3′ terminal region of yeast $tRNA^{Tyr}$ and has 2′-*O*-methyl guanosine as its second nucleotide to avoid the formation of an elongated transcript (*10*). *In vitro* transcription of pre-tRNA was basically performed according to the method of Sampson and Uhlenbeck (*9*). After 2 h of incubation at 42°C for transcription, 300 pmol each of M1 RNA and C5 protein per ml of reaction mixture were added and the mixture was incubated at 37°C for a further 1 h. The resulting mature-size tRNA transcript was purified by 10% PAGE containing 7 M urea.

Aminoacylation Assay—Tyrosine-charging (tyrosylation) reactions to determine the kinetic constants for each $tRNA^{Tyr}$ were carried out at 30°C in 100 μl of reaction mixtures as described in (*2*) with slight modification. The initial rates of aminoacylation were determined by using different concentrations of $tRNA^{Tyr}$ (ranging from 16 nM to 2 μM) at fixed concentrations of tyrosine (55 μM), ATP (4 mM) and various concentrations of yeast TyrRS, which gave reasonable kinetics plots for determining the apparent kinetic constants.

RESULTS AND DISCUSSION

Preparation of Yeast $tRNA^{Tyr}$ and Its Derivatives by Transcription and In Vitro *Processing*—Since yeast $tRNA^{Tyr}$ has a pyrimidine-rich tract CUCUC at its 5′-terminus (Fig. 2A), the conventional method of *in vitro* transcription using T7 RNA polymerase (*9*) is not a practical choice for preparing such a tRNA or its variants. Therefore, we designed a method for efficiently preparing tRNA transcripts which have 5′-terminal sequences that are disfavored by T7 RNA polymerase. Details are described in "EXPERIMENTAL PROCEDURES," but the outlines of the method are as follows (see also Fig. 1). Genes for yeast $tRNA^{Tyr}$ or its mutants (G34C mutation to form amber suppressor tRNA and/or U4C mutation to restore the wobble base-pair to a normal Watson-Crick type base-pair) were prepared as precursor (pre-tRNA) forms that contain 27 extra nucleotides at the 5′-region so as to

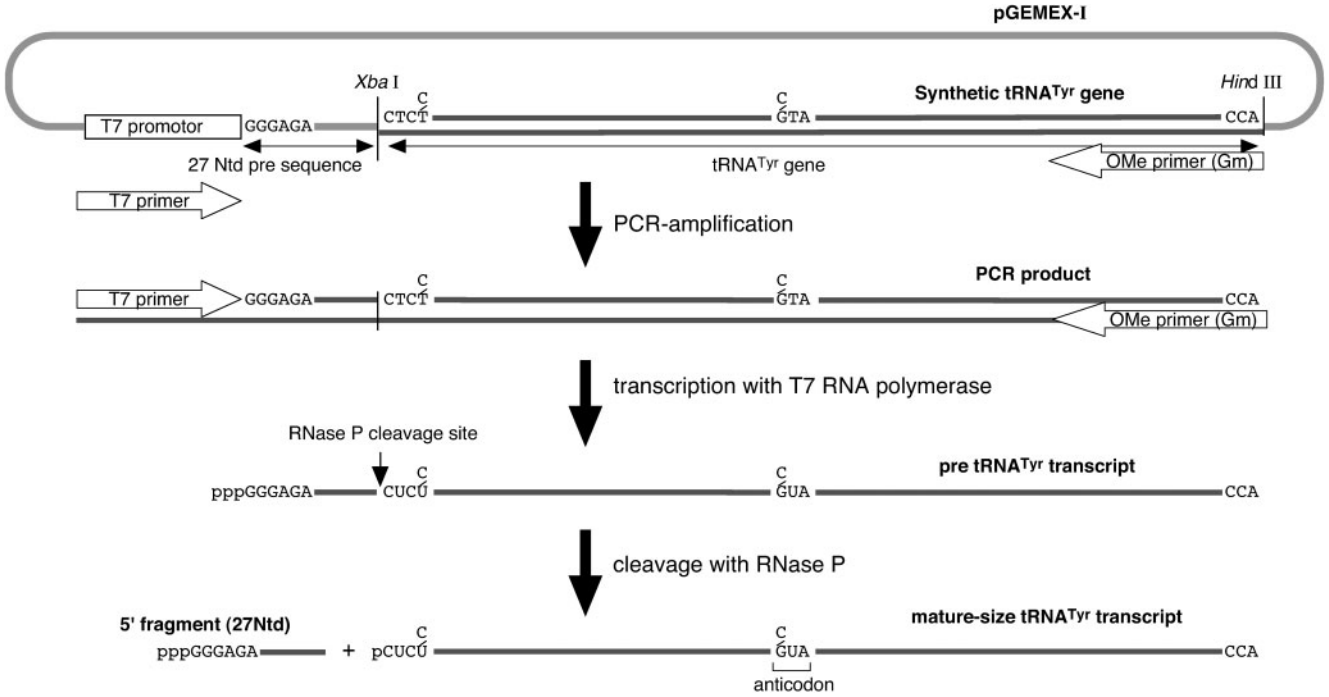

Fig. 1. **Procedure for preparing RNA transcripts having unfavorable sequences for transcription by T7 RNA polymerase.** Each of the synthetic genes for yeast $tRNA^{Tyr}$ or its mutants was ligated to the *Xba*I and *Hin*dIII sites of pGEMEX-1. This puts the genes directly downstream of the T7 promoter and an extra 27 nucleotides containing 5′-GGGAGA-3′, the sequence preferred by T7 RNA polymerase. Templates for transcription were prepared by PCR-amplification of this region by using a T7 primer and an OMe primer (see "EXPERIMENTAL PROCEDURES" for actual sequences). After 2 h of incubation at 42°C for transcription under the standard conditions (*9*), the pre-tRNA transcripts were processed with RNase P (reconstructed from purified M1 RNA and C5 protein) to generate the mature-size tRNA transcripts.

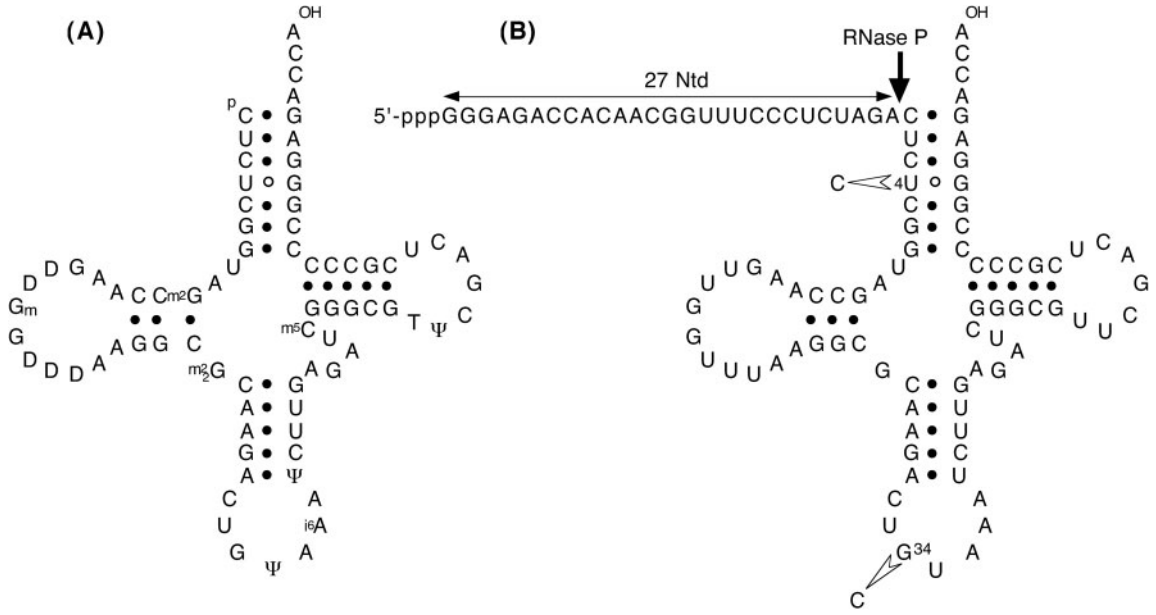

Fig. 2. **The nucleotide sequences of (A) yeast $tRNA^{Tyr}$ and (B) precursor transcripts of wild-type and mutant $tRNA^{Tyr}$s.** Sites of point mutation and RNase P cleavage are indicated by arrowheads and a bold arrow, respectively. Filled circles represent Watson-Crick base pairs, and open circles wobble pairs.

facilitate the transcription with T7 RNA polymerase (Fig. 2B). After transcription under the normal conditions, the transcripts (pre-tRNA) were processed with RNase P (reconstructed from purified M1 RNA and C5 protein) to generate the mature tRNAs free of modification (mature-size tRNAs). As shown in the typical PAGE pattern of pre-tRNA cleavage with RNase P (Fig. 3), more than 90% of the precursor tRNA was converted into the mature-size tRNA.

Reaction mixtures of 100 μl usually gave about 1.5 A_{260} units of final product tRNAs.

This method would greatly benefit those who wish to prepare tRNA or a related RNA having a pyrimidine-rich sequence at the 5′-end, since it is not efficiently produced by the most popular method of *in vitro* transcription using T7 RNA polymerase alone (*9*) and even if luckily produced in a small amount, the 5′-end of the product is mostly triphosphated. This method is also superior to the method of Giegé *et al*. (*11*, *12*) utilizing the hammerhead ribozyme in that the 5′-end of the product tRNA is phosphorylated as is native tRNAs and mutations in the acceptor stem region can be arbitrarily and easily introduced without taking the ribozyme architecture into consideration.

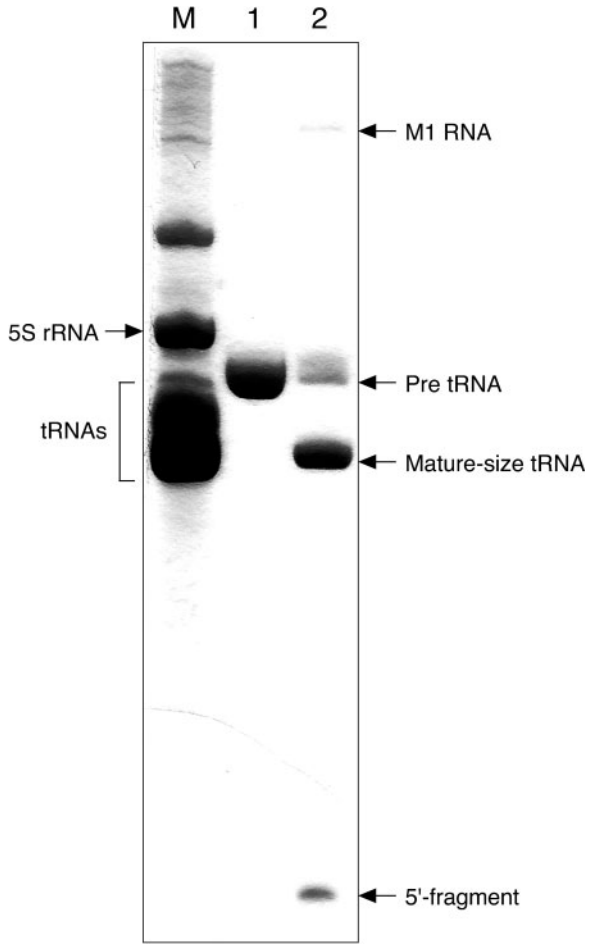

Fig. 3. **Cleavage of pre-tRNA transcripts with RNase P as analyzed by urea-PAGE.** M, *E. coli* total RNAs as molecular weight markers; 1ane 1, pre-tRNA transcript; lane 2. products of cleavage with RNase P.

Aminoacylation Properties of Yeast tRNA^{Tyr} Transcript and Its Mutants—Five kinds of yeast tRNA^{Tyr} or its derivatives were prepared in all. These include native yeast tRNA^{Tyr} (anticodon; GΨA), a transcript of wild-type tRNA^{Tyr} (anticodon; GUA), a tanscript of U4C mutant tRNA^{Tyr} (anticodon; GUA), a transcript of yeast amber supressor tRNA^{Tyr} (anticodon; CUA), and a transcript of amber supressor tRNA^{Tyr} harboring U4C mutation (G34C/U4C). Kinetic parameters for these tRNAs in the tyrosylation (tyrosyl-tRNA formation) reaction were determined as described in "EXPERIMENTAL PROCEDURES" and are summarized in Table 1.

The tyrosylation activity of wild-type tRNA^{Tyr} transcript is decreased to about 16% (794/4,915) of that of native tRNA^{Tyr}. This reflects the presence or absence of modified bases in the tRNA molecule in the recognition process by TyrRS. The activity of amber suppressor tRNA^{Tyr} is even lower. The G34 to C34 substitution results in about 1/794 reduction in the k_{cat}/K_m value (that for amber suppressor compared to that for wild-type transcript). This is in accordance with the study by Fechter *et al*. (*12*) in which a series of tRNA^{Tyr} variants harboring systematic replacements of single nucleotides within the molecule were prepared by the 'transzyme' method. They reported that G34C mutation (wild-type to amber-suppressor mutation) resulted in about 1/1,000 reduction in the k_{cat}/K_m of tyrosine-acceptance (*12*). Bare and Uhlenbeck constructed several anticodon variants of yeast tRNA^{Tyr} by the "microsurgery" method based on the partial digestion of native tRNA^{Tyr} with RNases and rejoining of the RNA fragments with RNA ligase (*13*). They reported that a substitution (G34 to C34) resulted in about a 9-fold reduction in V_{max}/K_m and double substitution (G34 to C34 and Ψ35 to U35) about a 37-fold reduction, although the effect of G34 to C34 substitution was less apparent due to the presence of modified nucleotides in these variant tRNAs.

We chose the U4:G69 wobble base-pair as the mutation target in the acceptor stem of yeast tRNA^{Tyr}, since this region had been the most troublesome part in which to introduce mutation by the method of Giegé *et al*. (*11*, *12*) utilizing the hammerhead ribozyme architecture, and it is known that the G3:U70 wobble base-pair in the acceptor stem of tRNA^{Ala} is a critical identity element of this tRNA (*14–16*). The overall yield of the U4C mutant or G34C/U4C double mutant was no less than that of tRNA^{Tyr} variants harboring mutation(s) in other part of the molecule (data not shown). This indicates that our method is virtually free of particular preference for the sites of mutation within

Table 1. **Kinetic parameters for tyrosylation of yeast tRNA^{Tyr} and its derivatives.**

tRNA^{Tyr}	(anticodon)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	Aminoacylation efficiency (χ-fold)[a]
Native	(GΨA)	0.071	3.13	44,085	4,915
Wild-type transcript	(GUA)	0.24	1.71	7,125	794
U4C transcript	(GUA)	0.15	2.17	14,467	1,613
G34C (amber suppressor) transcript	(CUA)	1.45	0.013	8.97	1
G34C/U4C transcript	(CUA)	0.12	0.010	16.1	1.8

[a]Aminoacylation efficiencies are expressed relative to the k_{cat}/K_m value for G34C (amber suppressor) transcript.

the tRNA molecule. In any case, yeast $tRNA^{Tyr}$ variants harboring a mutation in the acceptor stem region were prepared for the first time and their aminoacylation activity measured. As shown in Table 1, it is intriguing that the k_{cat}/K_m value of U4C-mutant transcript is about twice that of the wild-type transcript (14,467/7,125). This relationship is also true for the G34C/U4C double mutant and amber-suppressor (G34C-mutant) (16.1/9). This clearly indicates that the U4:G69 wobble base-pair in the acceptor stem of yeast $tRNA^{Tyr}$ is not a prerequisite for this tRNA in contrast to the case of $tRNA^{Ala}$. In fact, the mutant tRNAs with a C4:G69 normal Watson-Crick type base-pair (U4C mutant and G34C/U4C double mutant) are even better substrates for yeast TyrRS than the wild-type $tRNA^{Tyr}$. It is also notable that the $tRNA^{Tyr}$ from some species of yeasts like *Torulopsis utilis* (*17*) have a normal Watson-Crick type base-pair at this position. Yeast (*S. cerevisiae*) $tRNA^{Tyr}$ and *T. utilis* $tRNA^{Tyr}$ are both interchangeably aminoacylated with *S. cerevisiae* and *T. utilis* TyrRSs (K.N., unpublished result).

As described above, this method was devised for preparing tRNA transcripts that have 5′-terminal pyrimidine-rich sequences like yeast $tRNA^{Tyr}$. It proved to be very convenient and efficient once RNase P (C5 protein and M1 RNA) was prepared by overproduction of the *rnp*A gene product in *E. coli* cells and *in vitro* transcription of *rnp*B gene, respectively. We have prepared more than 20 mutants of yeast $tRNA^{Tyr}$ by this method and succeeded in optimizing the sequence so that it is not recognized by any other aminoacyl-tRNA synthetases than yeast TyrRS (Fukunaga *et al*., manuscript in preparation). This method will find more general use in preparing tRNA-like RNA strands that have sequences at their 5′-terminal region that are unfavorable to transcription by RNA polymerases including T7 RNA polymerase.

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