

# Nucleotides U28–A42 and A37 in unmodified yeast tRNA<sup>Trp</sup> as negative identity elements for bovine tryptophanyl-tRNA synthetase

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**Abstract** Wild-type bovine and yeast tRNA<sup>Trp</sup> are efficiently aminoacylated by tryptophanyl-tRNA synthetase both from beef and from yeast. Upon loss of modified bases in the synthetic transcripts, mammalian tRNA<sup>Trp</sup> retains the double recognition by the two synthetases, while yeast tRNA<sup>Trp</sup> loses its substrate properties for the bovine enzyme and is recognised only by the cognate synthetase. By testing chimeric bovine–yeast transcripts with tryptophanyl-tRNA synthetase purified from beef pancreas, the nucleotides responsible for the loss of charging of the synthetic yeast transcript have been localised in the anticodon arm. A complete loss of charging akin to that observed with the yeast transcript requires substitution in the bovine backbone of G37 in the anticodon loop with yeast A37 and of C28–G42 in the anticodon stem with yeast U28–A42. Since A37 does not prevent aminoacylation of the wild-type yeast tRNA<sup>Trp</sup> by the beef enzyme, a negative combination apparently emerges in the synthetic transcript after unmasking of U28 by loss of pseudourydilation. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Yeast tRNA<sup>Trp</sup>; Bovine tRNA<sup>Trp</sup>; Synthetic transcript; Tryptophanyl-tRNA synthetase

## 1. Introduction

Three cellular functions are well established for animal tRNA<sup>Trp</sup>: its participation in protein biosynthesis, its role in the initiation of DNA synthesis by avian retroviruses [1–3] and its function as cofactor of gelonin, a ribosome inactivating protein [4].

During the course of our work [5] on the identity elements of animal tRNA<sup>Trp</sup> required for the specific stimulation of gelonin, the casual observation was made that, in contrast to the well known fact that wild-type bovine and yeast tRNA<sup>Trp</sup> are both aminoacylated by the synthetase from bovine pancreas [6,7], the yeast synthetic tRNA<sup>Trp</sup> transcribed in vitro was not recognised by the bovine enzyme although efficiently charged by the aminoacyl-tRNA synthetase from yeast. The identity elements reported for the cognate synthetases in both native yeast and animal tRNA<sup>Trp</sup> have been located in the anticodon loop and stem [8,9]. Although the

identity elements might not be the same for the two tRNAs, apparently wild-type yeast tRNA<sup>Trp</sup> contains all the elements required for full recognition by both enzymes. The loss of recognition of the unmodified yeast tRNA<sup>Trp</sup> by the bovine enzyme might be explained either by the loss of a positive identity element involving a modified base or by the appearance of a negative identity element unmasked by the loss of modification, or by an instability of the tertiary structure of the synthetic tRNA following the loss of modification of a particular base [10,11].

In the present paper the structural features which make of the yeast transcript a very poor substrate for the bovine enzyme have been identified.

## 2. Materials and methods

The yeast unfractionated aminoacyl-tRNA synthetase preparation was from Sigma. Pure tryptophanyl-tRNA synthetase (TrpRS) from beef pancreas was obtained according to [12] except for the final gel-filtration step that was on a Sephacryl HR S-200. Purity of fractions was checked by SDS-PAGE in reducing conditions on 20% homogeneous gels run in a Phast System (Pharmacia).

Wild-type bovine tRNA<sup>Trp</sup> was purified from beef liver [13] and wild-type yeast tRNA<sup>Trp</sup> from brewer's yeast [14]. In both cases the presence of intact CCA ends was checked on 15% polyacrylamide gels. The choice of the source of yeast tRNAs came from the observation that 99% of brewer's yeast tRNA has an intact CCA end in contrast to baker's yeast tRNAs which in certain phases of the growth cycle show a pronounced degradation of the terminal CCA [15]. Bovine and yeast tRNA<sup>Trp</sup> transcripts were prepared as described in [5] by assemblage of the corresponding genes from synthetic oligonucleotides followed by PCR and T7 RNA polymerase transcription of the amplified products. Chimeric bovine–yeast tRNA<sup>Trp</sup> transcripts were synthesised using oligonucleotides designed according to the desired base substitutions. Upon electrophoresis on denaturing 20% polyacrylamide gels, all transcripts gave a single major spot. These spots were eluted, gel impurities were removed by chromatography on Mono-Q (HR 5/5, Pharmacia) and the purified transcripts, present in the Mono-Q 0.8 M NaCl eluate, were precipitated with ethanol, dissolved in water and stored in small aliquots at –80°C. At odds with their behaviour with beef TrpRS (see Figs. 1 and 3), all the transcripts used in our experiments retained the ability to accept tryptophan when assayed with the aminoacyl-tRNA synthetase preparation from yeast, indicating the presence of a correct CCA end. Charging of the chimeric transcripts by the yeast enzyme ranged from 45 to over 100% of the charging obtained with the wild-type yeast tRNA<sup>Trp</sup>. The variability was by no means related to the smaller or greater acceptor activity with the bovine TrpRS. Thus bovine tRNA<sup>Trp</sup> containing the yeast anticodon arm (which prevents aminoacylation by TrpRS) constantly showed with the yeast aminoacyl-tRNA synthetase an acceptance over 100% of that of wild-type yeast tRNA<sup>Trp</sup>.

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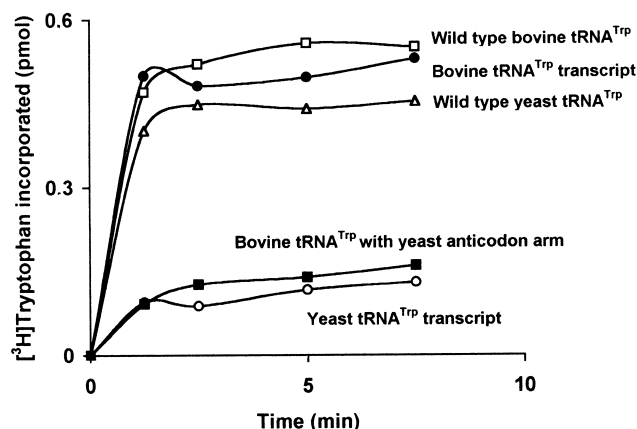


Fig. 1. Charging of wild-type yeast tRNA<sup>Trp</sup> and of tRNA<sup>Trp</sup> transcripts by bovine TrpRS. Triplicate experiments with two different preparations of the synthetic tRNAs gave consistent results. The aminoacylation assay is described in Section 2.

The standard aminoacylation assay [5] contained, in 25  $\mu$ l reaction mixtures, 100 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10 mM ATP, 1  $\mu$ Ci of [<sup>3</sup>H]tryptophan (30 Ci/mmol, Amersham), 0.24  $\mu$ M tRNA, 90 nM bovine TrpRS (or, alternatively, 40 U of yeast aminoacyl-tRNA synthetase) and 15 mM magnesium acetate. The excess of magnesium over ATP is required for a good acceptance of tryptophan by yeast tRNA<sup>Trp</sup> [6]. After incubation at 37°C the acid insoluble radioactivity was measured. In Figs. 1 and 3 the total amount of [<sup>3</sup>H]tryptophan (pmol) incorporated in the assays is indicated.

Kinetic constants ( $K_m$  and  $k_{cat}$ ) were derived from measurements performed in the same aminoacylation assay with the following exceptions. With the bovine transcript and with the wild-type yeast tRNA<sup>Trp</sup> as substrates, the enzyme concentration was reduced 10-fold (9 nM) so that the rate of the reaction was linear during the first minute of incubation at all concentrations of tRNA tested (0.12–1.92  $\mu$ M). With the yeast transcript and with the chimeric transcripts which similarly behaved as poor substrates for TrpRS, measurable rates of aminoacylation required the higher concentration of enzyme (90 nM), the tRNA concentrations ranged from 0.48 to 3.84  $\mu$ M, and the time of incubation (1 min) was such that the initial velocity was assessed.

<sup>32</sup>P-Labelled transcripts were prepared by adding trace amounts of [ $\alpha$ -<sup>32</sup>P]ATP to the T7 polymerase transcription reaction containing

1 mM of each rNTP [5]. The labelled transcripts were used to visualise by autoradiography the tRNA transcripts after native gel electrophoresis performed to evaluate the tertiary folding of the molecules. Electrophoresis was in the presence of 5 mM MgCl<sub>2</sub> as described in [10].

### 3. Results and discussion

The results in Fig. 1 confirm some of the data previously reported [5] and obtained with a crude preparation of bovine liver aminoacyl-tRNA synthetase. Wild-type yeast tRNA<sup>Trp</sup> and bovine unmodified tRNA<sup>Trp</sup> are both aminoacylated by purified bovine TrpRS, while recognition is lost with the yeast synthetic transcript in which modified bases are not present. In order to identify the structural elements involved in the loss of recognition, we have constructed several bovine tRNA<sup>Trp</sup> transcripts in which we have introduced yeast nucleotides and these transcripts were assayed for aminoacylation by the bovine enzyme. The accepting activity of bovine transcripts in which the four arms were separately changed to the yeast sequence clearly showed that substitutions in the acceptor stem, in the D stem, or in the T stem and loop only slightly affected (approximately 20% reduction) the charging activity (data not shown). In contrast, the presence of yeast nucleotides in the anticodon arm and at the adjacent position 44 induced a drop in activity akin to that observed with the synthetic yeast tRNA<sup>Trp</sup> (Fig. 1).

This result was not entirely surprising, since it is well known that the positive identity elements involved in the recognition of both bovine and yeast tRNA<sup>Trp</sup> by the cognate synthetases lie in the anticodon arm [8,9]. However, while the wild-type bovine and yeast tRNAs<sup>Trp</sup> conserve sufficient identity for recognition by both synthetases [6,7], such double recognition is lost when yeast unmodified nucleotides are present in the anticodon arm.

It is generally assumed that base modifications do not play a major role as positive elements in the acceptor activity of tRNAs, but rather introduce negative elements that block recognition by other aminoacyl-tRNA synthetases thus reducing the possibility of mischarging [16,17]. A particular role is

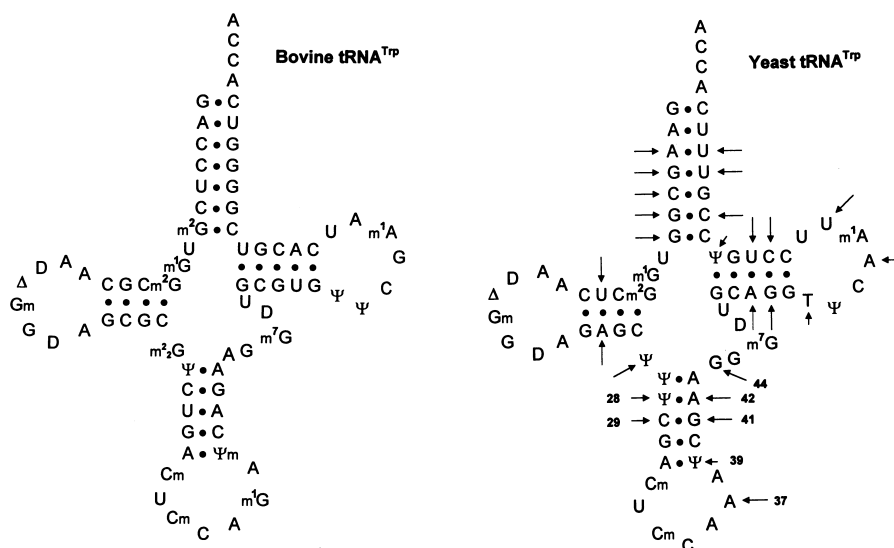


Fig. 2. Cloverleaf structure of wild-type bovine and yeast tRNA<sup>Trp</sup>. The primary structure of bovine tRNA<sup>Trp</sup> contains the most abundant nucleotide at heterogeneity points [7]. Arrows indicate nucleotides differing in the two tRNAs and short arrows the nucleotides disappearing in the synthetic transcripts. Numbers indicate the position of nucleotides differing in the anticodon arm and  $\Delta$  the absence of nt 17 in tRNA<sup>Trp</sup>.

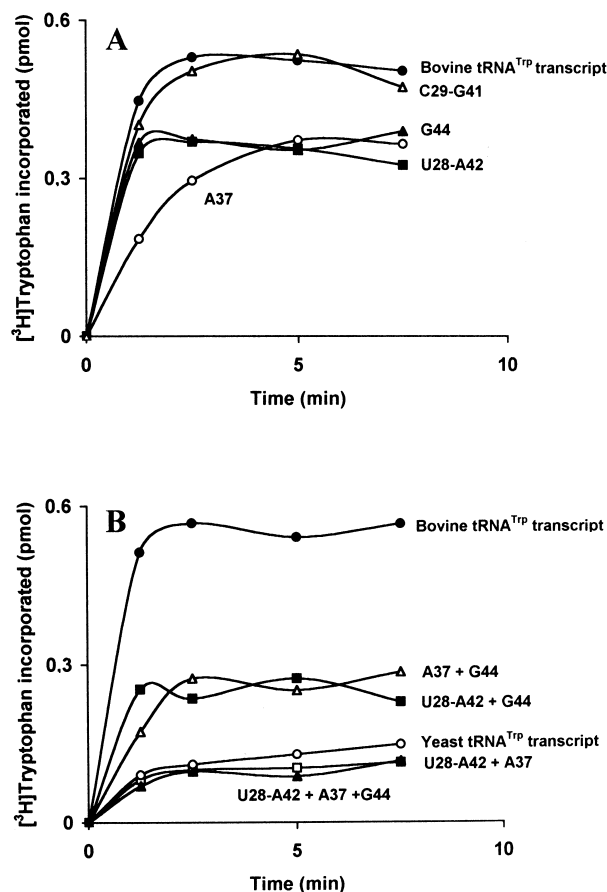


Fig. 3. Aminoacylation by bovine TrpRS of the bovine tRNA<sup>Trp</sup> transcripts carrying yeast nucleotides C29–G41, U28–A42, G44 and A37 compared with the aminoacylation of the two non-chimeric bovine and yeast transcripts. A: Substitutions were introduced at single positions. B: Substitutions were combined as indicated. Triplicate experiments with two different preparations of each transcript gave consistent results. The aminoacylation assay is described in Section 2.

ascribed to pseudourine ( $\Psi$ ), the most frequently modified nucleotide in tRNAs. Pseudouridylation in the arm stems stabilises the otherwise weak UA base pairs [11].

The anticodon arms of wild-type bovine and yeast tRNA<sup>Trp</sup> differ, in the stem, for the 2 bp at positions 28–42 and 29–41 and, in the loop, for the single stranded (ss) nucleotide adjacent to the anticodon at position 37 (see Fig. 2). A further difference at position 39 ( $\Psi$  or  $\Psi^m$ ) can be overlooked in the present discussion since it disappears in the unmodified synthetic tRNAs. Because of the boundaries chosen in separating the tRNA molecule in its four arms, bovine transcripts with

the yeast anticodon contained also ss G instead of ss A at position 44. Among these nucleotides only the one at position 28 ( $\Psi$ ) is modified in yeast tRNA<sup>Trp</sup>.

Fig. 3A shows the results obtained when the above yeast bp and ss nucleotides were separately introduced in the bovine backbone. Substitution of bp 29–41 (UA  $\rightarrow$  CG) had no effect on the charging efficiency of bovine TrpRS, whereas substitutions of bp 28–42 (CG  $\rightarrow$  UA) or of ss 44 (A  $\rightarrow$  G) induced a moderate loss of activity. Finally, the same final charging efficiency, but achieved at a much slower rate, followed substitution of ss 37 (G  $\rightarrow$  A).

An effect of bp 29–41 having been ruled out, substitutions of bp 28–42 and of the two ss nucleotides were differently combined. As shown in Fig. 3B, charging was only partially reduced by the combined substitution of bp 28–42 and ss 44 or by that of ss 37 and ss 44. It decreased instead to the level observed with the yeast transcript following substitutions at positions 28–42 and 37, both in the presence of yeast and bovine ss 44. Accordingly, the  $k_{\text{cat}}/K_m$  ratios measured with these substrates were, as that of the yeast transcript, 15-fold lower than the value obtained with wild-type yeast tRNA (Table 1). The 15-fold drop in the specificity constant  $k_{\text{cat}}/K_m$ , which indicates the substrate efficiency for a given enzyme [18], is in the higher range observed for unmodified tRNAs in respect to fully modified tRNAs [19]. Taken together, the results clearly indicate in yeast bp U28–A42 and ss A37 the major elements responsible for the inability of the yeast transcript to be recognised by the bovine TrpRS.

A negative influence of U28–A42 and A37 is in agreement with the observation that, in bovine tRNA<sup>Trp</sup>, besides the two cytidines at positions 34 and 35, the m<sup>1</sup>G residue 37 and positions 27–30 are the regions mostly protected from chemical reagents in the presence of the cognate TrpRS [9]. In the yeast transcript, the presence of G37 instead of the bovine G37 and a loosening of the anticodon stem because of the presence of bp UA instead of the more stable bovine bp CG, might well account for the inability of the tRNA to interact with the bovine enzyme. The profound effect of posttranscriptionally modified bases on the stability of the tRNA tertiary structure is well documented [10,11]. In the wild-type yeast tRNA<sup>Trp</sup> pseudouridylation at position 28 (UA  $\rightarrow$   $\Psi$ A), together with the two upstream pseudouridines 26 and 27, may be essential to the stability of the anticodon stem. The importance of these three consecutive pseudouridines is highlighted by the observation that hypomodifications at these positions were never found in sequencing work [20]. Although all the tRNA<sup>Trp</sup> transcripts not recognised by the bovine TrpRS (see Figs. 1 and 3) migrated as tertiary folded structures when assayed [10] in native gel electrophoresis in the presence of  $\text{Mg}^{2+}$  (data not shown), the result does not ex-

Table 1  
Aminoacylation kinetics with bovine TrpRS of wild-type yeast tRNA<sup>Trp</sup> and of yeast–bovine tRNA<sup>Trp</sup> transcripts

tRNA	$k_{\text{cat}}/K_m$	Relative
	( $\mu\text{M}^{-1} \text{min}^{-1}$ )	
Wild-type yeast tRNA <sup>Trp</sup>	11.63	1
Bovine tRNA <sup>Trp</sup> transcript	13.46	1.16
Yeast tRNA <sup>Trp</sup> transcript	0.76	0.06
Bovine tRNA <sup>Trp</sup> transcript with yeast U28–A42 and A37	0.83	0.07
Bovine tRNA <sup>Trp</sup> transcript with yeast U28–A42, A37 and G44	0.93	0.08

The decrease of the specificity constant of the last three transcripts is the consequence of an increase of  $K_m$  and of a decrease of  $k_{\text{cat}}$  of approximately the same magnitude (4-fold).

clude that an intrinsic instability of the anticodon region might show up during its interaction with the non-cognate TrpRS. Alternatively, pseudouridine 28 may be directly involved in the recognition process of the tRNA with the bovine enzyme.

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