

Construction, aminoacylation and 80 S ribosomal complex formation with a yeast initiator tRNA having an arginine CCU anticodon

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The digestion of yeast initiator methionine tRNA with mung bean nuclease and U₂ ribonuclease yielded 5'- and 3'-fragments, respectively. These two fragments together represent the entire tRNA sequence except for A₃₅, the central nucleotide of the anticodon, and the CCA terminus. Using RNA ligase, a cytosine was added and the anticodon loop having a C₃₅ was reformed. Subsequent treatment of this product with CCA-transferase yielded a full-length methionine tRNA having an arginine CCU anticodon. This recombinant tRNA^{Met} (CCU) was charged with methionine by the yeast tRNA synthetase. Aminoacylation of the recombinant was however less extensive than in the case of native tRNA^{Met} (CAU). After aminoacylation the recombinant tRNA formed an 80 S ribosomal complex.

initiator tRNA recombinant RNA Aminoacylation Ribosomal complex

1. INTRODUCTION

The initiation of protein synthesis is a complex phenomenon relying on specific interactions between a variety of proteins and nucleic acids among which, a unique methionine tRNA (tRNA^{Met}) plays a key role. This initiator species incorporates methionine at the N-terminal position of proteins in response to an initiation codon (generally AUG), while another tRNA^{Met} species incorporates methionine at internal positions [1]. Comparison of all initiator tRNA sequences from different organisms demonstrates highly conserved regions that distinguish this tRNA family from all others [2]. These regions are: parts of the T-loop,

D-loop and the anticodon loop and stem. We are attempting to relate a combination of these unique features to the initiation role of tRNA^{Met}.

Our method for delineating functional regions of this tRNA involves the use of structurally modified tRNA. To avoid the problems of expression in eukaryotic systems, these modifications are constructed at the RNA level. We report here the synthesis and some properties of a yeast initiator tRNA^{Met} having an arginine CCU anticodon. The choice of this modification is directed by the facts that prokaryotic tRNA^{fMet} (CAU) initiates protein synthesis at codons other than AUG [1,3] and that Schulman and Pelka [4] find that this particular variant has some activity in aminoacylation using *E. coli* methionyl-tRNA synthetase.

2. MATERIALS AND METHODS

Saccharomyces cerevisiae, haploid strain M₁-240a, was a generous gift from Dr Y.S. Chung of the Biology Department of the Université de Montréal. Aminoacyl-tRNA synthetases from

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baker's yeast (Sigma) was enriched in methionine charging activity by gel filtration on Sephadex G-25. Yeast CCA-nucleotidyl transferase was prepared by the technique of Sternbach et al. [5]. Purification of yeast tRNA_i^{Met} was achieved by consecutive BD-cellulose chromatography, DEAE-Sephadex A-50 chromatography, RPC-5 chromatography, 15% PAGE-urea gel electrophoresis and BD-cellulose chromatography of the phenoxyacetylated methionyl-tRNA_i^{Met}. The final purity was 1200 pmol/A₂₆₀ in 1.4% yield.

2.1. Aminoacylation

tRNA acceptor activity was assayed (after optimization of different factors, i.e. ATP/Mg²⁺, buffer and time) in 250 μ l of a mixture containing 50 mM Na cacodylate buffer (pH 7.5), 12.5 mM MgCl₂, 2.5 mM ATP and CTP, 8.6 μ M [¹⁴C]methionine (Amersham, 60.4 mCi/mmol) and 25 mM KCl. Incubation was for 10 min at 37°C using 100 units of the commercial enzyme/A₂₆₀ of tRNA. Aminoacylation of the reconstituted [³²P]tRNA was done in 25 μ l of the above mixture containing 35 μ M of either methionine, [¹⁴C]methionine (60.4 mCi/mmol) or [³⁵S]methionine (1600 Ci/mmol), 1 μ g *B. megatorium* 5 S RNA carrier, 10 μ g bovine serum albumin and 0.3 A₂₈₀ of methionyl-tRNA synthetase for 1–16 nM [³²P]tRNA (25000–100000 cpm). Incubation was for 20 min at 37°C. Phenoxyacetylation of [³²P]tRNA was performed as described [6,7].

2.2. Partial hydrolysis of tRNA with mung bean nuclease and U₂ RNase

The 5'-tRNA fragment (pA—C₃₄OH) was prepared by hydrolysis of 50–150 μ g purified tRNA_i^{Met} with 0.2–0.6 units mung bean nuclease (PL Biochemicals)/ μ g tRNA in 125 μ l of 30 mM Na acetate buffer (pH 4.5), containing 50 mM NaCl, 1 mM ZnSO₄ and 5% glycerol. After 15 min incubation at 37°C, the reaction was stopped by freezing in an ethanol-dry ice bath. The 3'-tRNA fragment (HOU₃₆—A₃p) was a product of partial U₂ RNase hydrolysis: samples of 150 μ g tRNA were submitted to digestion by 1 unit U₂ RNase (Sankyo)/ μ g tRNA in 500 μ l of 0.1 M Na acetate buffer (pH 5.5) containing 5 mM MgCl₂ for 15 min at 0°C. Purification of both fragments was accomplished on 20% polyacrylamide gel elec-

trophoresis in a 0.1 M Tris-Boric acid buffer (pH 8.3) containing 7 M urea and 1 mM EDTA. Bands containing the fragments were extracted with 125 μ l of 0.5 M ammonium acetate solution containing 1 mM MgCl₂, 0.1 mM EDTA and 0.1% SDS [8] for 18 h and the oligonucleotides were ethanol-precipitated. Identity of these fragments was confirmed by end-group analysis using T₂ RNase or P₁ nuclease (both enzymes from Sigma) and complete sequencing performed using partial enzymatic hydrolysis with T₁, U₂, Phy M and *B. cereus* RNases (all enzymes from PL Biochemicals) [9].

2.3. Reconstitution of anticodon-substituted tRNA

The reconstitution of tRNA_{ACC}^{Met} was accomplished according to the scheme in fig.1. To the 5'-fragment (500 ng, 40 pmol, 5.7 μ M) (pA—C₃₄OH) was added 75 pmol [³²P]pCp by treatment with 4 units T₄ RNA ligase (PL Biochemicals) for 24 h [10] at 4°C. This labelled fragment was 3'-dephosphorylated using 2.8 units T₄ polynucleotide kinase (PL Biochemicals) in 10 μ l of a buffer containing 100 mM imidazole-HCl (pH 6.0), 10 mM MgCl₂, 10 mM β -mercaptoethanol and 0.2 μ g bovine serum albumin; incubation was for 2 h at 37°C [11]. A sample of 400 ng (64 pmol) of the 3'-tRNA fragment (HOU₃₆—A₃p) was phosphorylated using 5 units T₄ polynucleotide kinase and 150 pmol [γ -³²P]ATP (3000 Ci/mmol) for 30 min at 37°C. These labelled fragments (5' and 3') were then purified on 20% polyacrylamide gels: the bands were extracted as before and the products ethanol-precipitated without added carrier RNA.

Each of the labelled fragments was then redissolved in 3.5 μ l ligation buffer [10], combined and annealed by incubation of the sample for 3 min at 60°C and decreasing temperatures (60 $\rightarrow$ 4°C) over a 1 h period. The reaction volume was completed by addition of ATP to a final concentration of 12 μ M, DMSO to 10% and 5 units T₄ RNA ligase and incubated for 16 h at 4°C. The reaction mixture was then acidified by addition of Na acetate buffer to 200 mM (pH 4.5), and ethanol-precipitated at -80°C for 90 min. The recovered tRNA was again subjected to 3'-dephosphorylation as described above and the CCA terminus of the [³²P]tRNA was added by

15 min incubation at 37°C in 10 μ l solution consisting of 50 mM glycine-NaOH buffer (pH 9.4), 2 mM CTP, 5 mM ATP, 10 mM MgCl₂ and 4 units purified yeast CCA-nucleotidyl transferase. The products were ethanol-precipitated and subjected to electrophoresis at 1700 V for 18 h on a 7 M urea-20% polyacrylamide gel. The product was excised from the gel and precipitated in the presence of 1 μ g *B. megatorium* 5 S RNA. The [³²P]tRNA was further desalted by filtration on a 1 ml column of Sephadex G-25 (medium) eluted with water. Identity of the anticodon was verified by nearest-neighbor analysis using total digestion with P₁ nuclease and T₂ RNase.

2.4. Formation of 80 S ribosomal complexes

Micrococcal nuclease-treated reticulocyte lysate samples were prepared [12] and supplemented with 20 μ g/ml of haemin (from Sigma), 5 μ g/ml of creatine phosphokinase (from Boehringer-Mannheim) and 50 μ g/ml of rabbit liver tRNAs. Ribosomal complex formation was performed in a final volume of 25 μ l consisting of 15 μ l of the lysate and 4 μ l of a solution to make a final concentration of 100 mM K acetate, 2.5 mM Mg acetate, 250 mM creatine phosphate and 20 μ M methionine-deficient amino acids. After 3 min incubation at 30°C, emetine (Sigma) was added to a final concentration of 0.1 mM and incubation continued for 5 min. A sample of 2.0 μ l Met-[³²P]tRNA (25 000–300 000 cpm) was then added and after 4 min, 1.5 μ l β -globin mRNA (BRL, 250 ng) or water was incubated for an additional 2 min. The reaction was stopped by dilution with 125 μ l of cold 10 mM Hepes buffer (pH 7.4), containing 75 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM EGTA and 0.2 mM spermidine [13]. The total volume was applied onto a 5 ml 15–40% sucrose gradient and centrifuged in a Beckman SW 50.1 rotor for 2 h at 48 000 rpm. 250 μ l samples were counted with 10 ml Triton-supplemented scintillant.

Wheat germ translation extracts were prepared according to Robert and Paterson [14] and stored in 20 mM Hepes buffer (pH 7.6) containing 90 mM K acetate, 3 mM Mg acetate, 0.1 mM phenylmethylsulfonyl fluoride (Sigma) and 6 mM β -mercaptoethanol. Ribosomal complex formation was performed as above in a final volume of 30 μ l consisting of 15 μ l wheat germ extract sup-

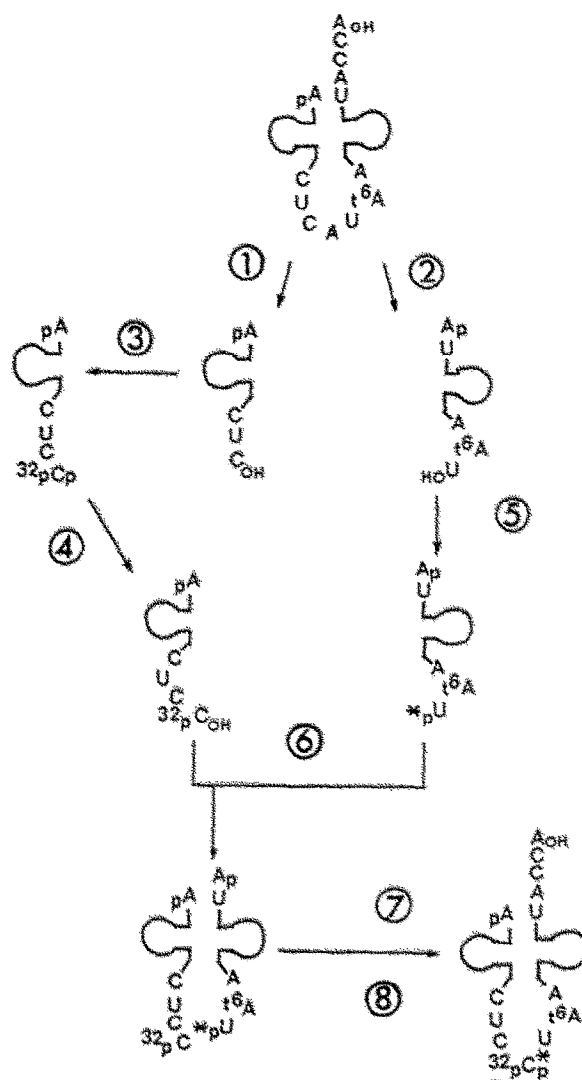


Fig.1. Reconstitution of tRNA^{Met}. tRNA^{Met} was hydrolysed under partial conditions described in section 2 by either mung bean nuclease (step 1) or U₂ RNase (step 2). To the 5'-tRNA fragment was added [³²P]pCp with the use of T₄ RNA ligase (step 3). This labelled fragment was then dephosphorylated at its 3'-terminus by T₄ polynucleotide kinase as described (step 4). In parallel, the 3'-tRNA fragment was phosphorylated using [γ -³²P]ATP and T₄ polynucleotide kinase (step 5). The two fragments were reannealed and ligated by T₄ RNA ligase (step 6). This truncated tRNA was again 3'-dephosphorylated (step 7) and repaired by CCA-nucleotidyl transferase (step 8). The asterisk indicates position of a second ³²P label in the anticodon.

plemented with 8.6 μ l of a mixture containing ATP (final concentration 1.4 mM), GTP (0.25 mM), creatine phosphate (15 mM), Hepes buffer (25 mM, pH 7.5), spermidine (0.4 mM), methionine-deficient amino acids (20 μ M), dithiothreitol (5 mM), creatine phosphokinase (40 μ g/ml) and calf liver tRNAs (40 μ g/ml). The concentrations of K^+ and Mg^{2+} were adjusted to 100 and 2.5 mM for translation of the β -globin mRNA.

3. RESULTS

3.1. Construction of a yeast tRNA^{Met} with an arginine anticodon

A prerequisite to the reconstruction as outlined in fig.1 was the isolation of pure 5'- and 3'-fragments of yeast tRNA^{Met}. This was accomplished by partial digestions by mung bean nuclease (5'-fragment) and U₂ RNase (3' frag-

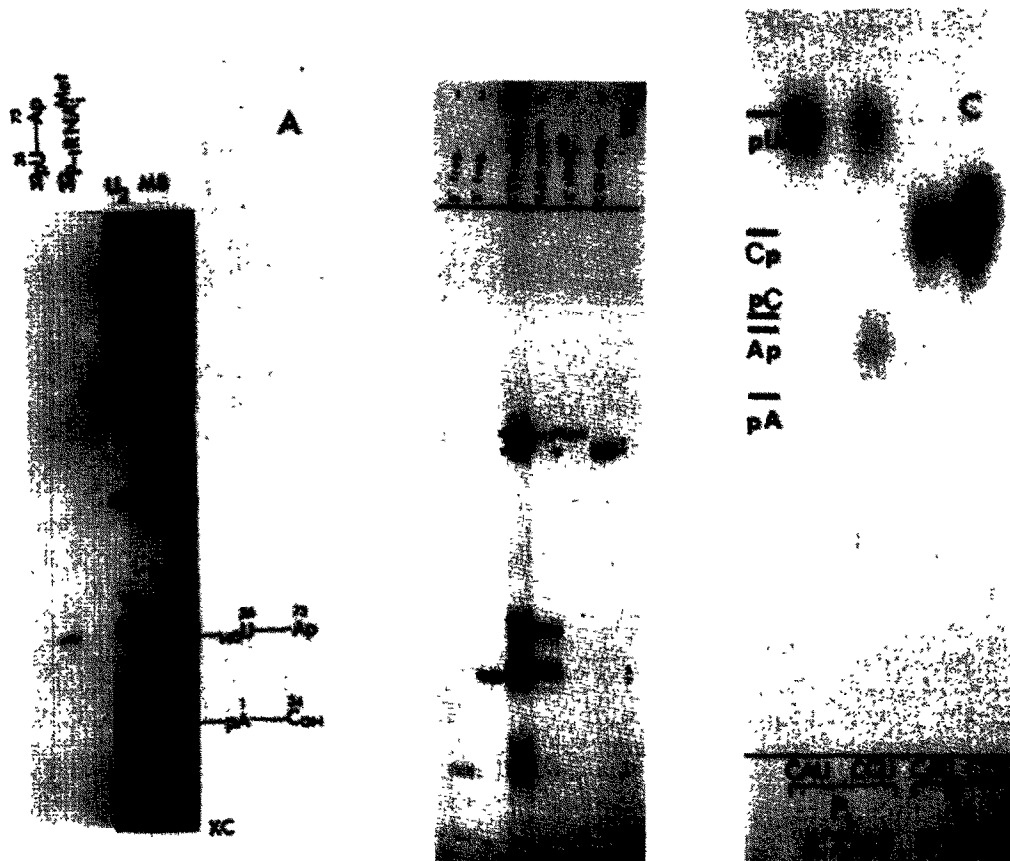


Fig.2. (A) Separation of mung bean nuclease and U₂ RNase partial hydrolysis products: two samples of 50 μ g tRNA^{Met} were digested with either 0.2 units/ μ g mung bean nuclease (MB) or 1.0 units/ μ g U₂ RNase (U₂) under the experimental conditions described in section 2. The hydrolysis products were then separated on a 7 M urea-20% polyacrylamide gel for 20 h at 1500 V. The left panel represents an autoradiogram of a gel separating 5'-[³²P]tRNA^{Met} and the [³²P]U₃₆-Ap fragment (3'-frag.). The right panel shows digestion products from mung bean nuclease (MB) and U₂ RNase (U₂) hydrolysis by UV shadowing, etc. XC, xylene cyanol dye. (B) Reconstitution of tRNA: samples recovered at the different steps described in fig.1 were analyzed on a 7 M urea-20% polyacrylamide gel for 18 h at 1750 V. Lane 1: 3'-labelled 5'-tRNA fragment after 3'-dephosphorylation (step 4). Lane 2: 5'-labelled 3'-tRNA fragment (step 2). Lane 3: final separation of reconstituted tRNA^{Accu} (step 8). Lane 4: final separation of reconstituted tRNA^{CAU}. Lane 5: 5'-labelled control tRNA^{Met}. Lane 6: religated fragments (step 6). (C) Mononucleotide analysis of tRNA^{Met} constructs: tRNAs were treated with P₁ nuclease and T₂ RNase and the products were analyzed by cellulose TLC developed in HCl-isopropanol-water (15:70:15) [21]. Positions of ³²P-labelled mononucleotides were identified by autoradiography and compared with those of unlabelled control nucleotides of known identity.

ment). Step 1 gave a 5% yield of the fragment $pA-C_{34}OH$ whereas in step 2, a 10% yield was obtained (fig.2A). [^{32}P]pCp was ligated to the 5'-fragment (step 3), and the 3'-phosphate was removed (step 4). Phosphorylation of the 5'-terminus of the fragment $HOJ_{36}-A_{33}p$ was accomplished by T_4 polynucleotide kinase. Reconstitution of the tRNA by T_4 RNA ligase, 3'-dephosphorylation of $pA-A_{33}p$ and addition of the CCA terminus were done to give the full-length tRNA^{Met}_{CCU} (fig.2B). Ligation of tRNA was efficient as shown in lane 6, since reprecipitated material of band 5 constituted 28% of the total radioactivity in that lane, the 5'- and 3'-fragments (bands 1-3) representing 5 and 3%, respectively. Repair of the 3'-terminus with yeast CCA-nucleotidyl transferase gave an 84% yield as seen in bands 11 and 13. The overall yield of reconstituted tRNA^{Met}_{CCU} (5.2 ng or 0.2 pmol) represented 1% of the original starting material.

For subsequent use as a control, small amounts of reconstituted ^{32}P -labelled tRNA^{Met}_{CAU} (with native anticodon) was synthesized by adding an A to the 3'-terminus of the 5'-fragment. Ligation with the 3'-fragment to produce the full-length tRNA (band 20 in lane 4) of fig.2B was much less effective than in the case of tRNA^{Met}_{CCU}, the limiting step being the initial addition of [^{32}P]pAp to the 5'-fragment.

The structure of the two tRNA constructs was verified by total hydrolysis with T_2 RNase and P_1 nuclease followed by TLC analysis. The tRNA^{Met}_{CCU} construct yielded radioactive pC and pU with P_1 nuclease and radioactive Cp with T_2 RNase. The reconstituted tRNA^{Met} (CAU anticodon) gave pA and pU with the former enzyme and Cp and Ap with the latter (fig.2C).

3.2. Aminoacylation of reconstructed tRNA

Given the nanogram levels of tRNA prepared according to the synthetic scheme outlined above, determinations of aminoacceptor activity were done by an indirect method, relying on the fact that phenoxyacetylated aminoacyl-tRNAs elute in a 20% ethanol-salt buffer in benzoylated DEAE-cellulose chromatography [6,7]. Uncharged or aminoacylated tRNAs were eluted anteriorly in a salt buffer. In this way, 45% of the native tRNA^{Met} was displaced in the ethanol buffer compared to 11% for the tRNA^{Met}_{CAU} construct (control

experiment) and 13% for the tRNA^{Met}_{CCU} construct. In addition, tRNA^{Met}_{CAU} and the reconstructed tRNA^{Met}_{CAU} gave values of 600 pmol/ A_{260} and 800 pmol/ A_{260} , respectively using [^{35}S]methionine in a standard aminoacylation assay. These values may in fact be underestimated due to the use of substrate concentrations (1-16 nM) lower than the K_m value (about 0.8 μM) [15]. The anticodon-substituted tRNA was not aminoacylated by an *E. coli* synthetase fraction, but was aminoacylated by the reticulocyte lysate or the wheat germ extracts used below in the tests of 80 S ribosomal complex formation. In addition, although this tRNA^{Met} contained the CCU arginine anticodon, no aminoacylation was found using arginine and a yeast enzyme preparation containing arginyl-tRNA synthetase (not shown).

3.3. Formation of 80 S ribosomal complex

A key question in our work concerned the ability of the reconstituted tRNA^{Met}_{CCU} to initiate protein synthesis by forming an 80 S ribosomal complex with other factors in the reticulocyte lysate and wheat germ extract systems. In both extracts, protein synthesis was blocked by emetine, an inhibitor of peptidyl-tRNA translocation [16]. Complex formation was assayed by sedimentation in sucrose gradients. The results are presented in fig.3. 80 S ribosomal complex formation was clearly evident in both extracts using native tRNA^{Met}_{CAU} and reconstruct tRNA^{Met}_{CCU}. The addition of [^{35}S]Met-[^{32}P]tRNA^{Met} allowed the observation that ^{32}P and ^{35}S coincided in the 80 S complex, confirming that the tRNA was aminoacylated and subsequently used in the 80 S ribosomal complex. Fig.3 also shows the absence of 80 S complex formation in the case of yeast tRNA^{Phe} (anticodon G_mAA) as expected of a true initiation complex dependent on the presence of initiator tRNA^{Met}_i. Table 1 summarizes these data.

Finally, a competition experiment was devised using tRNA^{Met}_{CCU} in the presence of native tRNA^{Met}_i and the wheat germ extract. The relative efficiency of the two tRNAs in 80 S ribosomal complex formation could not be easily evaluated since the native tRNA was labelled with ^{32}P at the 5'-terminal adenosine whereas the tRNA^{Met}_{CCU} contained two ^{32}P labels with different specific activity in the anticodon loop (respectively between nucleosides 33-34 and 34-35; see fig.1). Never-

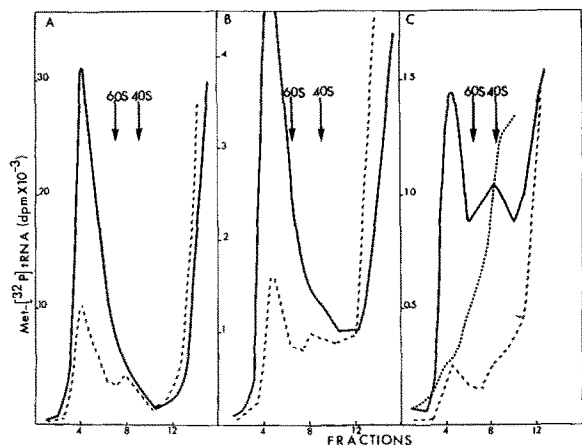


Fig.3. Formation of 80 S ribosomal complex. Emetine-arrested cell-free translation assays were performed as described in section 2. After 5 min incubation with emetine, samples of aminoacylated [^{32}P]tRNA were added and the incubation continued for 4 min; 250 ng β -globin mRNA were then incubated for an additional 2 min. The assays were then layered on 15–40% sucrose density gradients and centrifuged in a Beckman SW 50.1 rotor at 48000 rpm for 2 h. (A) Reticulocyte lysate assays: (---) Met-[^{32}P]tRNA $^{\text{Met}}$, (—) Met-[^{32}P]tRNA $^{\text{Met}}$. (B) Wheat germ extract assays: (---) Met-[^{32}P]tRNA $^{\text{Met}}$, (—) Met-[^{32}P]tRNA $^{\text{Met}}$. (C) Wheat germ extract assays: (...) [^{32}P]tRNA $^{\text{Phe}}$, (---) [^{35}S]Met, (—) [^{32}P]tRNA $^{\text{CCU}}$.

Table 1

Formation of 80 S ribosomal complex

Yeast tRNA used	Added [^{32}P]tRNA (ng)	[^{32}P]tRNA in complex (ng)
In reticulocyte lysate		
Met-tRNA $^{\text{Met}}$	1.7	0.57
Met-tRNA $^{\text{Met}}$	10.3	0.93
In wheat germ extract		
Met-tRNA $^{\text{Met}}$	6.7	0.13
Met-tRNA $^{\text{Met}}$	0.4	0.08
tRNA $^{\text{Phe}}$	1.3	0.003

theless, P_1 digestion of tRNA in the complex and the determination of the ratio of labelled nucleotides showed that the tRNA $^{\text{Met}}$ is at least as efficient as the native tRNA $^{\text{Met}}$ in forming the 80 S ribosomal complex.

4. DISCUSSION

In *E. coli* many modifications of the anticodon sequence have produced tRNA $^{\text{Met}}$ which cannot be aminoacylated [4,17,18]. However among these modifications the construct having a C $_{35}$ instead of A $_{35}$ can be aminoacylated with methionine to a measurable extent (i.e. 13% of the native tRNA), albeit at a considerably lower rate [4]. More recently, Schulman and Pelka [19] reported that reconstruction *E. coli* tRNA $^{\text{Met}}$ with anticodon CUA (instead of CAU), inactive for the *E. coli* methionyl-tRNA synthetase, was in fact partially aminoacylated by the glutaminyl-tRNA synthetase. We feel that the observed 10–15% displacement on BD-cellulose with our aminoacylated reconstituted tRNA $^{\text{Met}}$ or tRNA $^{\text{Met}}$ is therefore significant. Furthermore, the use of [^{35}S]methionine confirms that the construct can be aminoacylated to a reasonable extent (600 pmol/A $_{260}$), despite the fact that our experimental conditions were not optimal. This result certainly does not preclude a dramatic effect of the anticodon modification on the rate of aminoacylation in the eukaryotic system as it is for the prokaryotic system [4,17,18].

We have shown that the Met-tRNA $^{\text{Met}}$ is competent as well in the formation of the 80 S ribosomal complex. In addition, formation of an 80 S complex with this tRNA was observed in nuclease-treated reticulocyte lysate assays where addition of exogenous mRNA was omitted, a result probably due to the presence of equally small amounts of either undigested, endogenous mRNA or oligonucleotide fragments [20]. Also, the nanogram quantities of tRNA used in our experiment makes the quantitation of our results quite difficult. Nevertheless, the absence of complex with yeast tRNA $^{\text{Phe}}$, as well as the fact that our tRNA construct competes with native tRNA $^{\text{Met}}$ in 80 S ribosomal complex formation strongly suggest that a true initiation complex was formed. Given the specificity of the protein synthesis inhibitor (emetine) to block translocation of peptidyl-tRNAs [16] it seems likely that the tRNA $^{\text{Met}}$ can promote protein synthesis, but this remains to be demonstrated. The formation of the complex and presumably the initiation process are thus not affected by an A $_{35} \rightarrow$ C $_{35}$ substitution of the anticodon sequence in yeast initiator tRNA $^{\text{Met}}$.

However, we have no proof that the complex is indeed formed with an AUG initiation codon (experiments in progress). Recently, it has been suggested that a non-AUG initiation codon, which is recognized by the initiator methionyl-tRNA^{Met}, might be used in eukaryotic initiation [22]. Indeed, when a sequenced protein derived from the adeno-associated virus (AAV) was compared to the corresponding mRNA sequence, initiation would seem to start at an ACG threonine codon. This observation is relevant to the question of the importance of codon recognition by the methionyl-tRNA^{Met} during initiation of a eukaryotic messenger RNA.

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