Discrimination between initiation and elongation of protein biosynthesis in yeast: identity assured by a nucleotide modification in the initiator tRNA

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ABSTRACT
Cytoplasmic initiator tRNAs from plants and fungi possess an unique 2'-phosphoribosyl residue at position 64 of their sequence. In yeast tRNA<sub>Met</sub>, this modified nucleoside located in the T-stem of the tRNA is a 2'-1''-(β-O-ribofuranosyl-5''-phosphoryl)-adenosine. The phosphoribosyl residue of this modified nucleoside was removed chemically by treatment involving periodate oxidation of tRNA<sub>Met</sub> and regeneration of the 3'-terminal adenosine with ATP(CTP):tRNA nucleotidyl transferase. The role of phosphoribosylation at position 64 for interaction with elongation factor eEF-1α and initiation factor 2 (eIF-2) was investigated in the homologous yeast system. Whereas the 5'-phosphoribosyl residue prevents the binding of Met-tRNA<sub>Met</sub> to eEF-1α, it does not influence the interaction with eIF-2. After removal of the ribosyl group, the demodified initiator tRNA showed binding to eEF-1α, but no change was detected with respect to the interaction with the initiation factor eIF-2. This observation is interpreted to mean that a single modification of an eucaryotic initiator tRNA in yeast serves as a negative discriminant for eEF-1α, thus preventing the initiator tRNA<sub>Met</sub> from entering the elongation cycle of protein biosynthesis.

INTRODUCTION
In order to maintain the precision and accuracy of the translational apparatus, discrimination between the initiation and the elongation process of protein biosynthesis is essential. Translational initiation in procaryotes and in eucaryotic cytoplasm is accomplished by an initiator Met-tRNA<sub>Met</sub> recognizing the triplet initiation codon AUG. This process is assisted by the structure of mRNA and specific initiation factors. In procaryotes, the binary complex of eIF-2-GTP interacts only with aminocylated initiator tRNA<sub>Met</sub> [1]. In eucaryotes, this is accomplished by eucaryotic initiation factor eIF-2 [2]. The procaryotic initiator tRNA<sub>Met</sub> is excluded from entering the elongation process since EF-Tu, a protein responsible for the delivery of aminocylated elongator tRNAs to the ribosomal A-site is not able to form ternary complex with Met-tRNA<sub>Met</sub>. The reason for this is the unique structural elements of the initiator tRNA. Especially important is the missing first base pair 1–72 in the aminocyl stem of tRNA<sub>Met</sub> [3]. It was demonstrated that a mutation that restores this base pair directs tRNA<sub>Met</sub> to the elongation cycle without destroying its ability to participate in the formation of initiation complex. An additional structural element that prevents the bacterial initiator tRNA from entering the elongation cycle is the formulation of the α-amino group of methionine, which is specific for initiator Met-tRNA<sub>Met</sub>. Initiator tRNAs in eucaryotic cytoplasm do not undergo the formulation reaction. It is presumed that discrimination from elongation must be based on structural features distinct from that utilized by the procaryotic system. They are probably also located in the aminocyl- and the T-stems or in T- and the variable loops, the regions of tRNAs recognized by the bacterial elongation factor EF-Tu [4]. Additional structural features of eucaryotic initiator tRNA that play important discriminatory functions are the unusual A1-U72 base pair and the bases A54 and U55 which are uniquely occupied by ribothymidine and pseudouridine in elongator tRNA. In addition, nucleotides A20 and A60 may play a role in identifying tRNA<sub>Met</sub> during the process of translational initiation [5, 6]. The structure of 2'-phosphoribosyladenosine is shown in Figure 1. Occurrence of a modified purine nucleoside at position 64 in the T-stem of all initiator tRNAs in plants and fungi [7–10] prompted us to study the possible role of this modification as an initiation/elongation tRNA discriminatory element. The rationale for this hypothesis is based on the observation that the bacterial elongation factor EF-Tu forms a ternary complex with GTP and aminoacyl-tRNA in such a way that a large hydrophilic residue at position 64 could potentially interfere with the topology of EF-Tu·tRNA interaction. Indeed, native Met-tRNA<sub>Met</sub> from yeast interacts only weakly with the bacterial EF-Tu·GTP. Oxidative destruction and removal of the 5'-phosphoryl ribose from purine 64 improved the affinity of Met-tRNA<sub>Met</sub> to EF-Tu·GTP and formed ternary complexes with comparable affinities as bacterial elongator tRNAs [11]. We also demonstrated that demodification of A64 in yeast tRNA<sub>Met</sub> or G64 in wheat germ tRNA<sub>Met</sub>
directed these tRNAs to the elongation cycle, while the ability of these tRNAs to participate in the initiation reaction was retained [11]. A possible role of the yeast tRNA\textsubscript{Met} A64-phosphoribosylation for initiation/elongation discrimination has also been proposed by Byström and co-workers (personal communication). In this contribution we report the results of our investigation in the homologous yeast \textit{in vitro} system and the effect of A64 modification in yeast tRNA\textsubscript{Met} on its interaction with purified initiation factor eIF-2 and elongation factor EF-1\alpha from yeast.

**MATERIALS AND METHODS**

Yeast tRNA\textsubscript{Met} was isolated from the bulk tRNA purchased from Boehringer (Mannheim, Germany). The 3′-terminus of tRNA was regenerated according to a previously published procedure [12]. Aminoacylation was performed with [\textsuperscript{14}C]-methionine (specific activity 50 Ci/mole) obtained from Amersham-Buchler (Braunschweig, Germany). All other chemicals and biochemicals were obtained from standard sources.

Yeast tRNA\textsubscript{m} was separated from yeast tRNA\textsubscript{Met} by chromatography on BD-Cellulose (Serva, Heidelberg, Germany). Aminoacylation of the initiator tRNA\textsubscript{Met} was carried out using a partially purified \textit{E. coli} methionyl-tRNA synthetase. The elongator tRNA\textsubscript{m} was aminoacylated with methionine using an S100 fraction from yeast extract. The esterification reactions were carried out in buffers containing 50 mM Hepes, pH 7.6, 300 mM KCl, 10 mM MgCl\textsubscript{2}, 5 mM ATP and 6 μM tRNA. Initiator tRNA\textsubscript{Met} and the elongator tRNA\textsubscript{m} were aminoacylated up to 1600 and 1500 pmol of methionine/\textmu M of tRNA, respectively. Yeast eIF-1\alpha from yeast \textit{S. cerevisiae} was isolated as described [13]. Purified eIF-2 from yeast was prepared according to the published procedures [14] and was kindly provided to us by Dr N.K.Gupta of the University of Nebraska, Lincoln, NE. Demodification of tRNA\textsubscript{Met} and analysis of the reaction products were performed as described [11]. For labeling tRNA\textsubscript{Met} with \textsuperscript{32}P-pCP a standard procedure was followed [15].

Electrophoretic separation of the native and the oxidized tRNA\textsubscript{Met} was carried out on boronate containing polyacrylamide gels as described previously [16]. Affinity chromatography on phenylboronate substituted resins (Affigel 601, Biorad, Munich, Germany) for complexation of cis diol containing nucleotides was performed using 1 ml columns. RNase T2 and RNase A digests of tRNA were applied to the column in a buffer consisting of 0.25 M sodium formate, pH 9.0 and were eluted with 0.25 M sodium formate, pH 3.5.

Ternary complex formation of [\textsuperscript{14}C]-labeled Met-tRNA with yeast elongation and initiation factors were carried out in reaction mixtures containing 50 mM Tris/HCl, pH 7.5, 100 mM KCl, 10 mM MgAc, 5 mM dithiothreitol, 1 mM GTP, 5% (v/v) glycerol, 1.8 mM creatine-phosphate and 0.16 mg creatine kinase as a source of GTP generating system, 0.8 μM aminoacyl-tRNA, 0.8−4 μM eEF-1\alpha or eIF-2. The reactions were carried out in a volume of 25−100 μl for 10 min at 30°C. After specified time of incubation, the reaction mixtures were filtered immediately over nitrocellulose membrane and washed once with ice cold washing buffer containing 20 mM Tris/HCl, pH 7.5, 10 mM MgAc, 100 mM NH\textsubscript{4}Cl. The filters were dried and the retained radioactivity was determined by counting samples in a scintillation counter.

Hydrolysis protection experiments for aminoacyl-tRNA were carried out under similar reaction conditions described above except that the concentration of elongation factor was 5-fold over aminoacyl-tRNA. The reactions were terminated with 1 ml of 10% (w/v) trichloroacetic acid. The precipitates were collected over Whatman 3 MM filter paper, washed twice with 1 ml 10% (w/v) trichloroacetic acid, and once with 1 ml ethanol, once with 1 ml diethyl ether. All washing procedures were carried out with ice-cold solutions. The radioactivity retained on the filters was determined by counting samples in a scintillation counter.

**RESULTS**

Cis-diol affinity gel electrophoresis and affinity column chromatography on boronate substituted polymers

In Figure 2a electrophoretic separation is shown of tRNA\textsubscript{Met} derivatives which differ only in their modification at position 64. The separation of the native and the demodified initiator tRNA\textsubscript{Met} was demonstrated using phenylboronate substituted polyacrylamide gels. Evidently the phosphoribosyl residue of the modified adenosine is exposed and interacts with the gel, causing a retardation of the tRNA migration. In comparison, the periodate oxidized tRNA\textsubscript{Met} lacking the cis-diol function on the modified nucleoside 64 migrates faster. In this case, the 3′-terminal adenosine is protected from periodate oxidation by blocking the cis-diol function with pCP. However, when the native tRNA\textsubscript{Met}}
is treated with periodate, both the phosphoribosylated adenosine at position 64 and the 3'-terminal ribose are converted to aldehydes. Consequently, the relative mobilities of the native and the oxidized tRNA differ as shown in Figure 2a and 2b. From the comparison of the mobility differences between the native and the periodate oxidized tRNA\textsubscript{Met}, it is evident that the 3'-terminal cis-diol contributes more to the retardation of the mobility than does the ribose at position 64. This probably is due to the repulsion of phosphates and the negatively charged phenylboronate which is not likely to occur at the 3'-terminus, but may take place in the vicinity of the T-stem.

In order to confirm that tRNA\textsubscript{Met} was oxidized by treatment with Na-metaperiodate, both the oxidized and the native tRNA\textsubscript{Met} were digested with a mixture of RNase T2 and RNase A. The products of nuclease digestion were separated on a boronate affinity column. The resulting nucleoside-3'-phosphates showed no affinity to the boronate-substituted column matrix (Figure 3a). On the other hand, the cis-diol containing adenosine 76 from the CCA-end and the 2'-phosphoribosylated ApG-nucleotide dimer originating from position 64/65 of tRNA\textsubscript{Met}, which is not accessible to nuclease digestion, are retarded on the boronate column. These materials are eluted under acidic condition at pH 3.5 (Figure 3a). The late eluting fraction from the boronate column was dephosphorylated by treatment with alkaline phosphatase (Figure 3b).

Table 1. Quantitative analysis of the extent of ternary complex formation by [\textsuperscript{14}C]Met-tRNA\textsubscript{Met} (native); [\textsuperscript{14}C]Met-tRNA\textsubscript{Met} (ox) and by [\textsuperscript{14}C]Met-tRNA\textsubscript{Met} with either yeast eIF-2 or EF-1\textalpha

<table>
<thead>
<tr>
<th></th>
<th>Elongator</th>
<th>Initiator (ox)</th>
<th>Initiator</th>
</tr>
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<tr>
<td>pmoles aa-tRNA in complex with eEF1\textalpha-GTP</td>
<td>3.0</td>
<td>3.1</td>
<td>1.3</td>
</tr>
<tr>
<td>pmoles aa-tRNA in complex with eIF2-GTP</td>
<td>0.6</td>
<td>2.0</td>
<td>2.2</td>
</tr>
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The reaction conditions are the same as in Figure 4 except a five-fold excess of EF-1\textalpha and eIF-2 was used in these analyses.

Figure 5. Hydrolysis protection of [\textsuperscript{14}C]Met-tRNA\textsubscript{Met} (O-O); [\textsuperscript{14}C]Met-tRNA\textsubscript{Met} (ox) (●-●) and [\textsuperscript{14}C]Met-tRNA\textsubscript{Met} (△-△) by yeast EF-1\textalpha-GTP. The experiments were carried in buffers containing 50 mM Tris/HCl, pH 7.5, 100 mM KCl, 10 mM MgAc, 5 mM dithiothreitol, 1 mM GTP, 5% (w/v) glycerol, 2 μM [\textsuperscript{14}C]methionyl-tRNA and 10 μM EF-1\textalpha [13].
with alkaline phosphatase and rechromatographed on a reverse-phase HPLC column. The nucleosides retained by the boronate column were characterized by HPLC chromatography to be adenosine and a dinucleotide monophosphate A(2'-ribose)pG (Figure 3b).

Interaction of native and demodified tRNA_{Met} with homologous translational factors isolated from yeasts

The biological function of the demodified yeast tRNA_{Met} (lacking A-64 phosphoribosyl residue) was studied by investigating its participation in the formation of ternary complex with GTP and the yeast elongation factor EF-1α. Results presented in Figure 4 demonstrate that both the native elongator Met-tRNA_{m} and the demodified initiator Met-tRNA_{Met} participate in the formation of the ternary complex with yeast EF-1α and GTP. The native initiator tRNA_{Met}, on the other hand, fails to participate in this reaction with EF-1α beyond the unspecific binding. Based on the extent of ternary complex formation by the demodified initiator tRNA_{Met} and the elongator tRNA_{m}, at different EF-1α concentrations, we conclude that the affinity constants of these tRNAs for EF-1α are very similar.

To further establish the discriminatory function of A64 modification of tRNA_{Met}, the formation of ternary complex was followed using both the yeast initiation factor eIF-2 and the elongation factor EF-1α. Results presented in Table 1 show that the native tRNA_{Met} participates poorly in forming ternary complex with EF-1α-GTP. Upon demodification, tRNA_{Met} (ox) acquires the ability to recognize the EF-1α-GTP and still retains its binding capability to initiation factor eIF-2. The elongator tRNA_{m}, on the other hand, forms ternary complex exclusively with EF-1α-GTP.

Finally, the interaction between EF-1α and the demodified Met-tRNA_{Met} was demonstrated by the hydrolysis-protection experiment. The labile ester linkage between the COOH group of the amino acid and the 3'-end (OH group) of tRNA is protected by EF-1α [13] and by EF-Tu [17]. Met-tRNA_{Met} is not protected in this reaction. Results presented in Figure 5 demonstrate the ability of EF-1α to afford maximum protection to the ester linkage in Met-tRNA_{Met}, moderate protection in the demodified Met-tRNA_{Met}, and minimal protection in native Met-tRNA_{Met}.

**DISCUSSION**

Despite the progress in identification and structure determination of modified nucleosides in tRNA, the biochemical function of minor nucleotides is poorly understood. Base modifications are considered important for fine-regulation of protein biosynthesis, like codon–anticodon interaction, maintenance of the reading frame, accuracy of translation or for stabilization of tRNA structure [18].

Discrimination between the initiation and the elongation processes of cellular protein biosynthesis is of crucial importance for regulation of translation. In the procaryotic initiator tRNA, the structural elements and the enzymatic reactions that assure the discrimination between the two processes are well determined [3]. In eucaryotes, although there are obvious structural differences between the initiator and elongator tRNAs, features responsible for the discrimination are not clearly identified.

As shown in this work, the initiator tRNA from yeast is probably prevented from entering the elongation cycle of protein biosynthesis by a covalent modification of the adenosine residue located in the middle of the coaxial aminoacyl-T-helix of the L-shaped tRNA structure. Modification of position 64 in the T-stem of tRNA by a bulky and charged phosphoribosyl residue influences the interaction between Met-tRNA_{Met} and the bacterial elongation factor EF-Tu [11]. As reported by Basavappa and Sigler [5], the phosphoribosyl residue lies in the minor groove of the tRNA, and its 2'3'-hydroxyl groups are exposed to the solvent. The accessibility of the cis-diol to chemical reagents and its reactivity to form complex with affinity materials confirm this observation. The importance of this exposed structural element becomes clear when we consider the biological role of this modification.

The participation of the native and A-64 demodified yeast tRNA_{Met} in the elongation cycle has been investigated in *in vitro* experiments using the homologous yeast system. As demonstrated, A64 demodified initiator tRNA_{Met} is able to act as an elongator tRNA and can form ternary complexes with yeast eEF-1α and GTP. Remarkable is the fact that tRNA_{Met} (ox) forms a complex with eEF-2-GTP and does not lose its function as an initiator tRNA [11]. It appears that removal of the modification at position 64 does not lead to an identity-switch, but the tRNA assumes a double-identity. tRNA_{Met} that lacks the A-64 modification is able to act as an initiator and as an elongator tRNA. Since the demodified tRNA is taken out of equilibrium from the [Met-tRNA_{Met} • eIF-2 • GTP] initiation complex by interaction with the elongation factor EF-1α, the process of initiation could be affected. Since EF-1α represents a major protein (~5 %) in a cell, the inability of initiator tRNA_{Met} to interact with EF-1α provides a mechanism to assure the availability of the ternary complex containing eEF-2-GTP • Met-tRNA_{Met} during translational initiation. Based on our results, we propose that the presence of A64 modification in tRNA_{Met} may be involved in regulating the initiation of protein biosynthesis. The results presented in this investigation suggest that recognition sites for initiation and elongation factors on the tRNA molecules differ. EF-Tu recognizes parts of the T-loop, T-stem, aminoacyl-stem and the variable loop. Sufficient structural data on elongation and initiation factors are not yet available to allow interpretation of the results of this work in structural terms.

The modified nucleoside present in plant and fungi cytoplasmic initiator tRNA appears to act as a negative discriminant for the eucaryotic elongation factor eEF-1α and possibly regulates the rate of translational initiation. Here, we have an example for a possible control mechanism provided by a covalent, posttranscriptional modification of tRNA. Phosphoribosylation at position 64 in plant and fungi initiator tRNAs replaces a sequence coded identity element usually used to determine the protein/nucleic acid interaction of tRNA [19]. It is remarkable that mammalian initiator tRNAs and other eucaryotic cells except fungi and plants do not have a modification at position 64. It is likely that the higher eucaryotes utilize an alternate reaction mechanism or different structural features of the initiator tRNA_{Met} in order to prevent the interaction of the initiator tRNA with EF-1α.

**REFERENCES**