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Recognition of UUN codons by two leucine tRNA species from *Escherichia coli*

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Abstract

Codon recognition by Escherichia coli $tRNA^{Leu}_4$ and $tRNA^{Leu}_5$ was investigated by analysis of the competition between two aminoacyl-tRNA species in an in vitro protein synthesis. Both tRNA species strictly obey the wobble rule when they are in competition with other tRNA species. This is probably due to the post-transcriptional modifications at the first position of the anticodon of these $tRNA^{Leu}$ species, supporting the proposal that the conformational rigidity of post-transcriptionally modified pyrimidine nucleotides guarantees the correct codon recognition.

Key words: Codon recognition; Modified nucleoside; Protein synthesis (in vitro); Aminoacyl-tRNA competition; Conformational rigidity; Restricted wobbling

1. Introduction

The wobble hypothesis is based on the possible hydrogen-bonding schemes between the third base of the codon and the first base of the anticodon of the tRNA [1]. Later, it was found that some post-transcriptional modifications at position 34 (the first position of the anticodon) regulate codon recognition by controlling the conformational rigidity and flexibility of the nucleotide residue at this position [2,3]. In *E. coli*, $tRNA^{Leu}_4$ and $tRNA^{Leu}_5$ are responsible for the recognition of the UUA and UUG codons, and at position 34, they have the post-transcriptionally modified nucleosides 5-carboxymethylaminomethyl-2′-*O*-methyluridine (cmnm^{5}Um) and 2′-*O*-methylcytidine (Cm), respectively [4]. Both of these modified nucleosides are conformationally rigid [4,5]. Therefore, it was suggested that these modifications also diminish the formation of nonorthodox base pairs with the third base of the codon [4].

The codon recognition properties of these leucine tRNA species have been studied by in vitro protein synthesis [4,6,7], because bindings of these tRNA species to *E. coli* ribosome were not significantly stimulated by a UUA triplet [8]. The results indicated, however, that $tRNA^{Leu}_5$ reads the UUA codon efficiently, and recognizes A as the third letter by the use of Cm at position 34.

In the present study, we have quantitatively analyzed codon recognition by $tRNA^{Leu}_4$ and $tRNA^{Leu}_5$ in an in vitro protein synthesis by controlling the relative amounts of the aminoacyl-tRNA species. This allowed us to determine the relationship between the codon recognition properties of these tRNA species and the dynamic conformational properties of the modified nucleosides at the first position of the anticodon.

2. Materials and methods

2.1. Construction of plasmids

The chloramphenicol acetyltransferase (CAT) gene from plasmid pBR325 [9] was modified, and the sequence of the mRNA coding region is summarized in Fig. 1. The CAT gene of pACL1 and the internally

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Abbreviations: CAT, chloramphenicol acetyltransferase; CAT′, internally deleted CAT; Cm, 2′-*O*-methylcytidine; cmnm^{5}Um, 5-carboxymethylaminomethyl-2′-*O*-methyluridine; HEPES, *N*-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); mnm^{5}s^{2}U, 5-methylaminomethyl-2-thiouridine; N, the four nucleosides A, C, G, and U; Tris, tris(hydroxymethyl)aminomethane.

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deleted CAT (CAT') gene of the pACL7 series were used for in vitro transcription-translation.

2.2. Preparation of E. coli *extract (S30) for coupled transcription-translation*

E. coli A19 cells [10] were grown in 2 × YT medium [11], harvested when the A_{600} reached 4, and homogenized by sonication. The subsequent preparation of S30 extract from the cell lysate was as described [12].

2.3. Preparation of aminoacyl-tRNAs

E. coli $tRNA^{Leu}_4$ was purified from a tRNA mixture from strain A19 by chromatography on columns of DEAE-Sephadex A50 (pH 7.5) [13,14] and benzoylated DEAE-cellulose [15]. *E. coli* phenylalanine tRNA was eluted as an isolated peak in the same benzoylated DEAE-cellulose chromatography. As for $tRNA^{Leu}_5$, the preparation obtained in our previous study [4] was used.

These tRNA species were aminoacylated by incubation for 15 min at 37 °C in a reaction mixture containing 160 mM Tris-HCl (pH 7.2), 10 mM 2-mercaptoethanol, 10 mM magnesium acetate, 10 mM potassium chloride, 10 mM ATP, 5–10 μg/ml tRNA, one-fifth volume of S100 extract prepared as described [13], and the cognate amino acid. Tritium-labeled leucine (592 GBq/mmol) was used for the aminoacylation of tRNA species whose activities were to be measured, while ^{14}C-labeled leucine of a low specific activity (252 MBq/ mmol) or non-radioactive phenylalanine was used for aminoacylation of the competitor tRNA.

2.4. Analyses of codon recognition by tRNAs

The reaction mixture (10 μl) contained 55 mM HEPES-KOH, pH 7.0, 1.7 mM dithiothreitol, 1.2 mM ATP, 0.85 mM each of CTP, UTP, and GTP, 27 mM phospho-*enol*-pyruvate (potassium salt), 0.9 mM leucine, 0.37 mM each of the other 19 amino acids, 1.9% polyethyleneglycol 6000, 35 μg/ml folinic acid (calcium salt), 0.64 mM 3',5'-cyclic AMP, 0.17 mg/ml tRNA from *E. coli* MRE600 (Boehringer-Mannheim), 36 mM ammonium acetate, 90 mM potassium acetate, 9.7 mM calcium acetate, 8–11 mM magnesium acetate, 0.1 mg/ml plasmid DNA, 0.31 volume of S30 extract, and various amounts of aminoacyl-tRNAs.

For analyses of synthesized proteins, samples were electrophoresed through Tris-Tricine SDS polyacrylamide gels [16], in which ethylene diacrylate was used as the crosslinker [17]. Gels were cut into segments of equal lengths so that the radioactivity could be scanned along the lanes. Each gel segment was dissolved in 1 M NH_4OH [17] and its ^{3}H radioactivity was counted using ReadyCap (Beckman) and a liquid scintillation counter LSC-700 (Aloka).

3. Results and discussion

In this study, three plasmids were used for analyses of codon recognition. Each plasmid was used for a particular codon. Plasmid pACL1 was used for the analysis of codon UUA, pACL7TTC45 was used for the phenylalanine codons, and pACL7TTG45 was used for codon UUG.

3.1. Recognition of codon UUA by $tRNA^{Leu}_5$

As shown in Fig. 2, the incorporation of [^{3}H]leucine from Leu-$tRNA^{Leu}_4$ with cmnm^{5}Um, or Leu-$tRNA^{Leu}_5$ with Cm was measured for the reaction mixtures using plasmid pACL1, which encodes the CAT mRNA with three UUA codons and no UUG. Leucine incorporation was efficient and was not affected by Leu-$tRNA^{Leu}_5$ (Fig. 2c,d). On the other hand, incorporation from Leu-$tRNA^{Leu}_5$ into the CAT was not observed when equal amounts of Leu-$tRNA^{Leu}_4$ and Leu-$tRNA^{Leu}_5$ were in competition (Fig. 2a). In contrast, leucine was incorporated from Leu-$tRNA^{Leu}_5$

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 1                                  10                                  20
Met Glu Lys Lys Ile Thr Gly Tyr Thr Thr Val Asp Ile Ser Gln Trp His Arg Lys Glu
AUG GAG AAA AAA AUC ACU GGA UAU ACC ACC GUU GAU AUA UCC CAA UGG CAU CGU AAA GAA

 21                                  30                                  40
His Phe Glu Ala Phe Gln Ser Val Ala Gln Cys Thr Tyr Asn Gln Thr Val Gln Leu Asp
CAU UUU GAG GCA UUU CAG UCA GUU GCU CAA UGU ACC UAU AAC CAG ACC GUU CAG CUG GAU

 41              45                  50                                  60
Ile Thr Ala Phe Leu Lys Thr Val Lys Lys Asn Lys His Lys Phe Tyr Pro Ala Phe Ile
AUU ACG GCC UUU UUA AAG ACC GUA AAG AAA AAU AAG CAC AAG UUU UAU CCG GCC UUU AUU
                ↓
               UUC (pACL7TTC45)
               UUG (pACL7TTG45)

 61                                  70          73  74        158       207 208
His Ile Leu Ala Arg Leu Met Asn Ala His Pro Glu Phe Arg ..... Leu ..... Glu Leu
CAC AUU CUU GCC CGC CUG AUG AAU GCU CAU CCG GAA UUC CGU ..... UUA ..... GAA UUA

209                                 218 219
Gln Gln Tyr Cys Asp Glu Typ Gln Gly Gly Ala
CAA CAG UAC UGC GAU GAG UGG CAG GGC GGG GCG UAA
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Fig. 1. Coding sequences of the CAT and the CAT' mRNAs from plasmids pACL1 and pACL7, respectively. Approximately two thirds of the coding sequence, indicated by a thick bar (from the 74th codon to the 208th codon), was deleted to construct pACL7 from pACL1. The UUA leucine codon (number 45) is altered in mRNAs from plasmids pACL7TTC45 and pACL7TTG45.

into CAT, when Leu-$tRNA^{Leu}_4$ was omitted from the reaction and only Leu-$tRNA^{Leu}_5$ was added (Fig. 2b). Therefore, $tRNA^{Leu}_5$ has the potential to recognize codon UUA, but it is too low to be detected under competitive conditions between $tRNA^{Leu}_5$ and $tRNA^{Leu}_4$. In the previous study [6], $tRNA^{Leu}_5$ was shown to read codon UUA about half as efficiently as $tRNA^{Leu}_4$. This is probably because Leu-$tRNA^{Leu}_4$ was supplied as the deacylated tRNA contained in the S30 extract at only a relatively low concentration, which was not sufficient to diminish the UUA-recognition by Leu-$tRNA^{Leu}_5$.

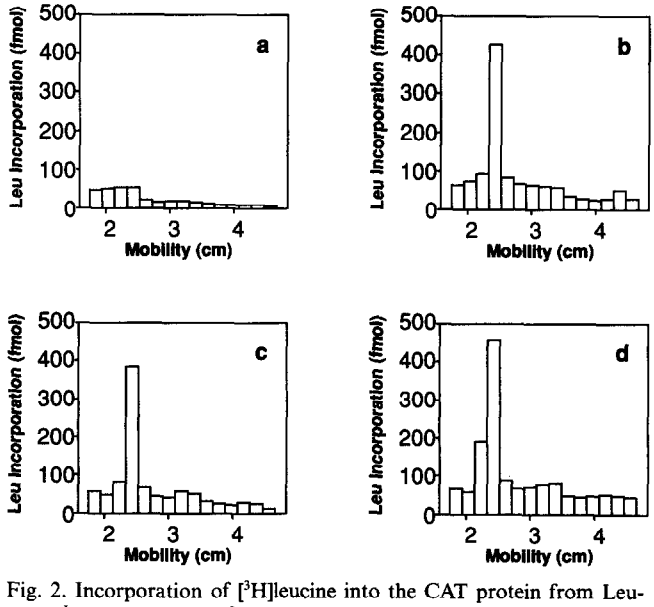

Fig. 2. Incorporation of [^{3}H]leucine into the CAT protein from Leu-$tRNA^{Leu}_4$ and Leu-$tRNA^{Leu}_5$. The CAT mRNA includes three UUA and lacks UUG codons. The reaction mixtures were supplemented with: (a) 10 μM [^{3}H]Leu-$tRNA^{Leu}_5$ and 10 μM non-radioactive Leu-$tRNA^{Leu}_4$ (see text); (b) 10 μM [^{3}H]Leu-$tRNA^{Leu}_5$; (c) 10 μM [^{3}H]Leu-$tRNA^{Leu}_4$ and 10 μM non-radioactive Leu-$tRNA^{Leu}_5$; (d) 10 μM [^{3}H]Leu-$tRNA^{Leu}_4$.

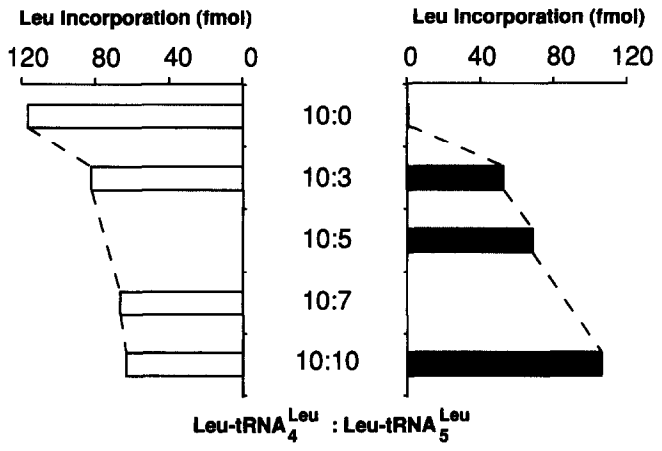

Fig. 3. Recognition of codon UUG by $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$. The ratio of Leu-$tRNA_5^{Leu}$/Leu-$tRNA_4^{Leu}$ added to the reaction is indicated in the middle. Incorporation into the CAT′ protein is compared using different concentrations of Leu-$tRNA_5^{Leu}$ along with 1.3 μM Leu-$tRNA_4^{Leu}$ and 5 μM Phe-tRNA, except for the reaction with a Leu-tRNA ratio of 10:5, where 2.7 μM Leu-$tRNA_4^{Leu}$ and 10 μM Phe-tRNA were included. The white bar (left side) indicates Leu-$tRNA_4^{Leu}$ is ^{3}H-labeled; the shadowed bar (right side) indicates Leu-$tRNA_5^{Leu}$ is ^{3}H-labeled. The incorporation shown in this figure was calculated from the sum of the radioactivity in two gel fragments containing the CAT′ protein minus that in the adjacent two fragments.

3.2. *Unorthodox recognition of codons terminating in A by tRNAs with either C or a modified C at position 34*

It has been shown that the C·A base pair is detectable in the codon–anticodon interaction [6,18] and in an artificial anticodon–anticodon interaction [3]. *E. coli* $tRNA_1^{Gly}$, with the anticodon CCC, recognizes codon GGA even in the presence of an equimolar amount of the competitor $tRNA_2^{Gly}$, with the anticodon UCC [18]. In contrast, it was shown here that the interaction between Cm at the first position of the anticodon and A at the third position of the codon is detectable, but much weaker than that between cmnm^{5}Um and A (Fig. 2b).

3.3. *Recognition of codon UUG by $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$*

Recognition of codon UUG was measured at different Leu-$tRNA_5^{Leu}$/Leu-$tRNA_4^{Leu}$ ratios (Fig. 3). Plasmid pACL7TTG45 was used to direct protein synthesis. The mobility of the synthesized protein in the gel electrophoresis was as expected from the molecular weight of the CAT′ protein with a deletion. In Fig. 3, the background radioactivities in the gel segments have been subtracted. Thus, both tRNA species were shown to recognize codon UUG, in agreement with the previous study [6]. The relative efficiency between the two tRNA species can be estimated from the values in the competition of the test tRNA with the same amount of the competitor [18–22]. The efficiency of $tRNA_5^{Leu}$ in reading codon UUG was found to be higher than that of $tRNA_4^{Leu}$ (Fig. 3).

3.4. *Recognition of codons terminating in G by tRNA species with differently modified uridines at position 34*

Both modifications of U(34), to cmnm^{5}Um(34) in $tRNA_4^{Leu}$ and to mnm^{5}s^{2}U(34) (mnm^{5}s^{2}U = 5-methylaminomethyl-2-thiouridine) in the glutamine, lysine, and glutamate tRNAs from *E. coli* confer conformational rigidity to the anticodons, and have been proposed to prohibit misrecognition of codons terminating in U or C [2,4]. One of the differences between the coding properties of cmnm^{5}Um and mnm^{5}s^{2}U is in the efficiency of recognition of codons terminating in G. It has been suggested that tRNAs with mnm^{5}s^{2}U(34) can read codons terminating in G only weakly [22–24]. The results in Fig. 3, on the other hand, show that the anticodon cmnm^{5}UmAA can contribute substantially to the reading of codon UUG, even when in competition with $tRNA_5^{Leu}$.

3.5. *Misreading of phenylalanine codons by $tRNA_4^{Leu}$*

The CAT′ mRNA derived from pACL7TTC45 includes five UUU codons and two UUC phenylalanine codons, and lacks UUA and UUG codons. Using this

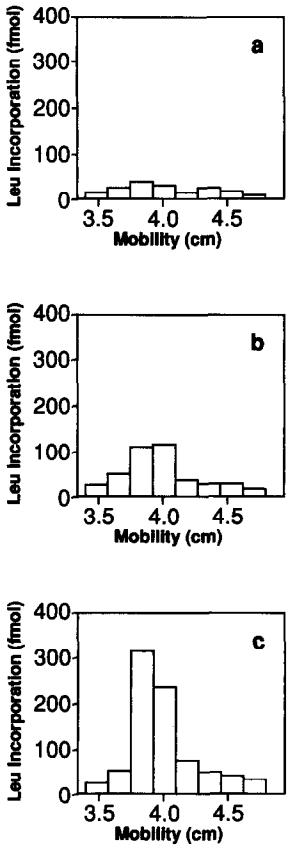

Fig. 4. Misincorporation of [^{3}H]leucine into the CAT′ protein during protein synthesis directed by plasmid pACL7TTC45. (a) 1.3 μM [^{3}H]Leu-$tRNA_4^{Leu}$ + 4 μM Phe-tRNA; (b) 1.3 μM [^{3}H]Leu-$tRNA_4^{Leu}$ with no supplemented phenylalanine; (c) 1.3 μM [^{3}H]Leu-$tRNA_4^{Leu}$ + 0.35 mM phenylalanine.

mRNA, we measured leucine misincorporation (Fig. 4). When nonradioactive Phe-tRNA was supplemented, no incorporation of leucine was observed (Fig. 4a). When Phe-tRNA was omitted, a low level of misincorporation of [^{3}H]leucine into polypeptides with the size of the CAT′ protein was observed (Fig. 4b). When phenylalanine was omitted from the reaction mixture, in order to lower further the level of Phe-tRNA, the level of misreading increased (Fig. 4c). The major portion of the synthesized protein was the size of the CAT′ protein, probably because complete depletion of phenylalanine could not be attained. Under phenylalanine starvation conditions, some of the phenylalanine codons have been reported to be translated as leucine in vivo [25,26]. An extremely low level of Phe-tRNA may cause mistranslation. Accordingly, it is concluded that $tRNA^{Leu}_4$ has the potential activity to read phenylalanine codons, but it is too small to be exhibited in competition with $tRNA^{Phe}$. $tRNA^{Gly}_2$, with a mixture of uridine and some modified uridine at position 34, reads codons GGU and GGC efficiently, even when in competition with *E. coli* $tRNA^{Gly}_3$ with the anticodon GCC [18]. Thus, we conclude that the modification from U(34) to cmnm^{5}Um(34) contributes to the prevention of misreading by $tRNA^{Leu}_4$. It has been suggested that cmnm^{5}Um is a conformationally rigid, modified uridine [4], and it is reasonable that wobble recognition by $tRNA^{Leu}_4$ is restricted within UUA and UUG codons.

3.6. *Aminoacyl-tRNA competition and restricted wobbling due to conformational rigidity of the anticodon*

The present study shows, as predicted from the conformational properties of the modified nucleosides, that $tRNA^{Leu}_4$ reads codons UUA and UUG, and that $tRNA^{Leu}_5$ reads codon UUG under competitive conditions with each other and with $tRNA^{Phe}$. For *E. coli*, conformationally rigid, modified uridines at position 34 are found in all the tRNA species that should not recognize codons terminating in U or C [2,4,27]. A conformationally rigid, modified cytidine, N^4-acetylcytidine, is found at position 34 of the *E. coli* methionine tRNA, and this modification is also considered to be effective in preventing recognition of isoleucine codons [28,29]. Many other organisms have conformationally rigid, modified uridines and cytidines at position 34 of tRNA species that are only allowed to recognize codons terminating in purine for the correct translation of genetic information [30]. Conformationally rigid modifications may have evolved because aminoacyl-tRNA competition does not sufficiently prevent incorrect codon recognition.

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