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E. coli initiator tRNA analogs with different nucleotides in the discriminator base position

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ABSTRACT

The effect of base changes at the fourth position from the 3'-terminus of Escherichia coli initiator $tRNA^{Met}$ has been studied to test the 'discriminator hypothesis' which proposed that the nucleotide in this position might have a role in the specificity of the aminoacylation reaction. E. coli initiator tRNA lacking the 3'-terminal tetranucleotide was prepared by partial digestion with S1 nuclease. To construct tRNA analogs with different bases in the fourth position this truncated tRNA was joined by RNA ligase to each of four chemically synthesized 2',3'-ethoxy-methylidene tetranucleotides pACCA(em), pCCCA(em), pGCCA(em), and pUCCA(em). In vitro aminoacylation studies showed that all four molecules accepted methionine, albeit with different V_{max} values.

INTRODUCTION

The 'tRNA recognition problem', the question of what specific nucleotides in a tRNA molecule determine the specificity of the aminoacylation reaction (1,2), has fascinated biochemists for a long time. Ten years ago the 'discriminator hypothesis' was proposed, to subdivide the family of tRNAs into groups for recognition purposes (3). It was suggested that the nucleotide occupying the fourth position from the 3'-terminus of a tRNA could serve as a primary discriminator. Each such group would possess its own recognition code; tRNAs with similar base sequence but changes in the discriminator nucleotide may be charged with different amino acids. In the intervening years a large number of tRNA species from many different organisms have been sequenced (4). Comparison of their sequences shows that the discriminator nucleotide is generally, but not exclusively, conserved within an isoacceptor family in the same organism. In addition, mutant tRNA species with changes in the discriminator nucleotide are known, which possess different amino acid specificity (5,6).

Recent advances in the methodology of chemical and enzymatic synthesis of ribo-oligonucleotides have allowed the construction of relatively large RNA molecules. For instance, RNA species with the nucleotide sequence

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of E. coli initiator $tRNA^{Met}$ (7) or yeast alanine tRNA (8) have been constructed by joining chemically synthesized oligoribonucleotides with RNA ligase, and many different yeast $tRNA^{Phe}$ analogs (9) have been prepared by an enzymatic procedure (10). These approaches lend themselves to site directed mutagenesis of tRNA species, which should be useful in unraveling the molecular details of the functions of these molecules.

E. coli initiator $tRNA^{Met}$ possesses an adenosine in the discriminator position. We decided to construct analogs of this tRNA which contain different bases in the fourth position from the 3'-terminus (position 74) to determine the effect on aminoacylation by methionyl-tRNA synthetase. This was accomplished by joining with RNA ligase chemically synthesized tetranucleotides of the structure pNCCA to an initiator tRNA fragment lacking the terminal tetranucleotide.

MATERIALS AND METHODS

General. T4 RNA ligase was purified as described earlier (11). Polynucleotide kinase and E. coli alkaline phosphatase were gifts of M. Sugiura. ($-^{32}P$)ATP was prepared by the method of Walseth and Johnson (12). E. coli $tRNA_f^{Met}$ was a gift of Dr. A. Kelmers (Oak Ridge National Laboratory) and had a specific acceptor activity of 1.4 nmol/A_{260} unit. Polyacrylamide gel electrophoresis was carried out in the presence of 7 M urea (13).

Oligonucleotides. 2',3'-Ethoxymethylidene tetranucleosides of the structure pXCCA(em) were synthesized by first joining of p^1-adenosine 5'-p^2-2',3'-ethoxymethylideneadenosine, A(5')ppA(em), to trinucleoside diphosphates NpCpC (N=A,G,U and C) with RNA ligase (14) and then introducing a 5'-phosphate using polynucleotide kinase (15) and ($-^{32}P$)ATP of specific activity (50 cpm/pmole).

Nuclease S1 digestion of tRNA. E. coli initiator $tRNA^{Met}$ (100 A_{260} units) in 2 ml of 1 mM $ZnCl_2$ - 5 mM $MgCl_2$ was digested with nuclease S1 ($3x10^4$ units) at 37° for 2 hr. After phenol extraction, the partially digested tRNA was precipitated with ethanol (6 ml) and the RNA mixture separarated by polyacrylamide (10%) gel electrophoresis. The desired product, tRNA lacking the 3'-terminal tetranucleotide and designated $tRNA^{Met}$(S1), was isolated in about 30% yield.

Periodate oxidation of tRNA. E. coli $tRNA_f^{Met}$ (60 A_{260} units in 3 ml) was treated with 9 mM sodium periodate in 0.1 M sodium acetate (pH 5.2) at 37° for 4 hr. The solution was then made 0.5 M in lysine-HCl (pH 8.0) to cause base elimination (16). The oxidized tRNA was then treated with

alkaline phosphatase and purified by polyacrylamide gel (10%) electrophoresis. tRNA preparations obtained after one or two cycles of this process were used as electrophoresis markers.

RNA ligation, kinase and phosphatase treatment. Ligation of the chemically synthesized tetranucleotides (10 nmol) to the digested tRNA (4.7 nmol) was carried out in the presence of ATP (15 nmol) and RNA ligase (3.1 units) in 0.19 ml of 50 mM HEPES-NaOH (pH 8.3) - 10 mM MgCl - 10 mM DTT - 10% DMSO (v/v) at 25° for 2.5 hr. The enzyme was removed by two extractions with an equal volume of buffer saturated phenol. The phenol layer was washed three times with 50 mM Tris-HCl (pH 7.5) and the combined aqueous layes were washed with ether. The RNA product was passed through a Sephadex G-50 column (10ml) and the ethoxymethylidene group was removed by treatment with 0.02 N HCl (0.1ml) at 25° for 1 hr. The mixture neutralized with triethylamine and purified by polyacrylamide gel (12%) electrophoresis.

3'-End-labeling of tRNA with (5'-^{32}P)pCp (specific activity 1 Ci/mmol) and RNA ligase was performed as described earlier (15).

Phosphomonoester groups were removed by treatment with E. coli alkaline phosphatase (0.005-0.015 units/nmol substrate) in 50 mM Tris-HCl (pH 8.0) either at 37° for 3 hr, or at 55° for 33 min. The enzyme was inactivated by addition of nitrilotriacetic acid to a final concentration of 5 mM and incubation at 25° for 30 min, then at 100° for 2 min.

Aminoacylation reactions. They were performed with pure E. coli methionyl-tRNA synthetase (18) or a crude E. coli aminoacyl-tRNA synthetase preparation (19). The reaction mixture contained per ml: 100 mM HEPES-NaOH (pH 8.3), 10 mM KCl, 10 mM magnesium acetate, 10 mM 2 mercaptoethanol, 4 mM ATP, 0.009 mM (^{14}C)-methionine (specific activity 285 mCi/mmol), aminoacyl-tRNA synthetase, and 0.5 nmol of tRNA. After incubation at 37° acid insoluble radioactivity was measured by the filter paper method (20). For normal aminoacylation studies the concentration of methionyl-tRNA synthetase was 0.7 uM, while it was 7 nM for the K_m studies. In the latter case four independent determinations were performed for each experimental point.

RESULTS

Synthesis of the ethoxymethylidene tetranucleotides. The starting materials for the construction of the ethoxymethylidene tetranucleotides pNCCA(em) were unprotected trinucleoside diphosphates of the structure NpCpC (N=A,C,G and U) which were synthesized by the standard phosphodiester approach (21). They were extended at their 3'-end with 2',3'-ethoxymethyli-

dene adenosine 5'-phosphate using RNA ligase to form the tetranucleoside triphosphates NpCpCpA(em). The ethoxymethylidene group (em) is needed to prevent side reactions of the terminal 2',3'-hydroxyl groups in the subsequent RNA ligase condensation to form the tRNA analogs. The final step in the tetranucleotide synthesis, the 5'-phosphorylation, was accomplished with the aid of polynucleotide kinase and ($-^{32}$P)ATP of low specific activity.

Synthesis of four initiator tRNA species which differ in the nucleotide located in the 'discriminator position'. The construction of these modified tRNA molecules was accomplished by removal of the 3'-terminal tetranucleotide pApCpCpA from intact initiator tRNA followed by reattachment of the chemically synthesized tetranucleotides pNpCpCpA(em) by RNA ligase. This scheme is outlined in Figure 1, where the sequences of the molecules are shown. Although a number of methods are available to remove the 3'-terminal nucleotides of tRNA, (e.g., partial hydrolysis by venom phosphodiesterase (22) or chemical sequential degradation (16)) they were not completely satisfactory in our hands. It was shown earlier that nuclease S1 is useful in cleaving E. coli initiator tRNA uniquely in the anticodon loop (23). However, we found that with a small amount of enzyme, digestion could be limited to the removal of the 3'-terminal ACCA. This material was designated $tRNA^{Met}$ (S1). As Figure 2 shows, the material obtained from the nuclease S1 digest is homogeneous. Sequence analysis (Figure 3) proved that this product had the expected 3'-terminal sequence.

This RNA was then linked with the aid of RNA ligase to the four different tetranucleotides pNCCA(em), which were labelled at their 5'-end with

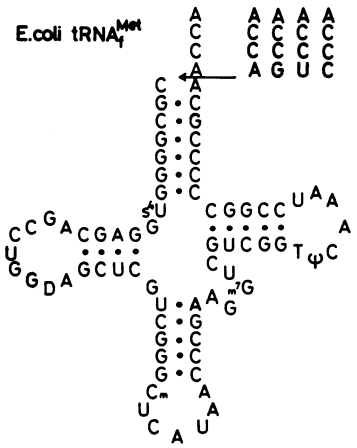

Figure 1.
Sequence of the E. coli initiator $tRNA^{Met}$ and the four chemically synthesized tetranucleotides. The arrow indicates the cleavage position of nuclease S1.

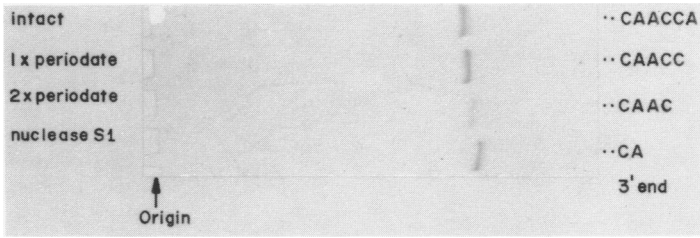

Figure 2.
Polyacrylamide (10%) gel electrophoresis of $tRNA^{Met}$(S1), intact tRNA, and tRNA shortened by one or two nucleotides by periodate treatment. 15 ug of each RNA was applied; detection was by staining with 0.2% acridin orange. The 3'-terminal sequence of the tRNA is indicated.

(^{32}P)-phosphate. After removal of the ethoxymethylidene group with dilute acid, the purity of the final preparation of the four modified tRNAs (designated $tRNA^{Met}$(S1)pNCCA) was checked by polyacrylamide gel electrophoresis. As viewed in Figure 4, the products are homogeneous and migrate with intact tRNA.

Aminoacylation of initiator tRNA analogs. The RNA analogs were tested for their methionine acceptor activity by in vitro aminoacylation with pure E. coli methionyl-tRNA synthetase. As can be seen from Table I

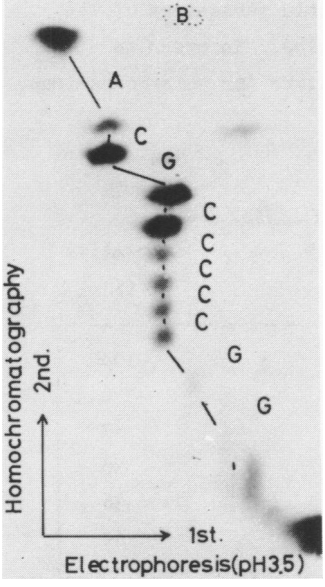

Figure 3.
Determination of the 3'-terminal sequence $tRNA^{Met}$(S1). (^{32}P)pCp labelled $tRNA^{Met}$(S1) was partially digested with nuclease P1 and separated by electrophoresis and homochromatography as described (27).

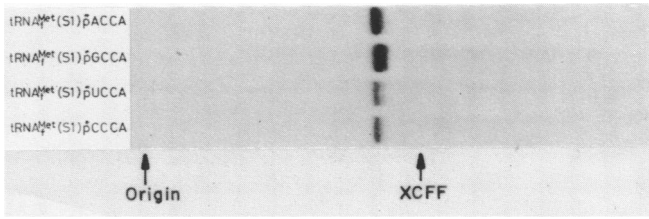

Figure 4.
Polyacrylamide gel (10%) electrophoresis of radioactive reconstructed tRNAs.
* denotes (^{32}P)-phosphate.

tRNA^{Met}(S1)pACCA, the reconstructed tRNA with the same sequence as the native molecule, has 63% of the acceptor activity of the untreated sample. This lower activity might be due to incomplete renaturation of the RNA after elution from the gel or to the presence of contaminants eluted from polyacrylamide with the RNA sample. As expected, the nuclease S1 treated tRNA$_f^{Met}$ lacking the 3'-terminal tetranucleotide did not have methionine acceptor activity, even when assayed in the presence of CTP with a crude cell extract fraction containing both terminal nucleotidyl transferase and methionyl-tRNA synthetase. As can be seen from Table I the three tRNAs modified to contain cytidine, guanosine and uridine in the 'discriminator' position can all be aminoacylated, albeit to different final levels. In order to examine the effects of each of the different nucleotides in this position in more detail the kinetic parameters of the aminoacylation reaction were determined for each analog. In order to conserve material we choose only our standard conditions for this comparison.

Table I
Aminoacylation Levels of Modified tRNAs.

tRNA	final level (pmol/A_{260})	Relative (%)
intact tRNA^{Met}	1400	100
tRNA^{Met}(S1)	0	0
tRNA^{Met}(S1)pACCA	878	63
tRNA^{Met}(S1)pCCCA	420	30
tRNA^{Met}(S1)pGCCA	504	36
tRNA^{Met}(S1)pUCCA	867	62

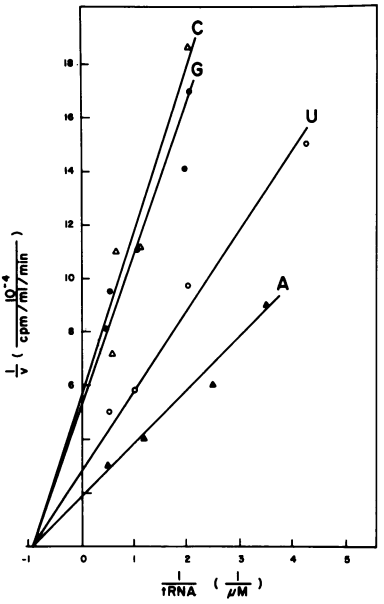

Figure 5.
Lineweaver-Burk plot for the amino-acylation of the $tRNA^{Met}$ analogs by pure E. coli methionyl-tRNA synthetase. For details see Materials and Methods. A,C,G and U denote $tRNA^{Met}$(S1)pACCA, $tRNA^{Met}$(S1)pCCCA, $tRNA^{Met}$(S1)pGCCA and $tRNA^{Met}$(S1)pUCCA, respectively.

The Lineweaver-Burk plot shown in Figure 5 indicates that the K_m values are the same for all four tRNAs. The value of 0.9 uM is in the range of values determined by Waller et al., for different ionic conditions (24). It is clear from the plot that the relative V_{max} values differ for four modified tRNAs. The modified tRNAs containing C and G in the discriminator position aminoacylate slowly and to lower levels under the same reaction conditions. This suggests that the nucleotide in position 74 is important for acylation of E. coli initiator $tRNA^{Met}$ by the cognate synthetase.

Since it is known that some mutant tRNAs with single base changes (compared to wild-type) can be misacylated (6,7), we wanted to know whether the methionine tRNA analogs could be charged with other amino acids. Therefore we tried to aminoacylate in vitro the intact and modified $tRNA^{Met}$ species with a mixture of 15 radioactive amino acids (excluding Asn, Cys, Gln, Met, Trp) using a crude E. coli aminoacyl-tRNA synthetase fraction. No miscylation was observed (data not shown) for the intact and modified tRNAs under the standard conditions. This result does not rule out possible mischarging in vivo since it has been shown in the past that in vitro mis-charging requires the use of partially purified or pure preparations of both synthetases and tRNA (25).

DISCUSSION

The most interesting result of this study is the finding that all four *E. coli* initiator $tRNA^{Met}$ analogs with changes in position 74 can be aminoacylated by the cognate aminoacyl-tRNA synthetase. This implies that the 'discriminator hypothesis' is not correct and that recognition, by the cognate synthetase is not critically dependent on the nucleotide in this position. This result was not unexpected, since it is known that the G82 mutant of *E. coli* su^{+3} $tRNA^{Tyr}$, which has a base change in the discriminator position, can still be charged with the cognate amino acid, presumably by the normal tyrosyl-tRNA synthetase (25). In addition, there are two cases known (the cytoplasmic $tRNA^{Lys}$ and $tRNA^{Arg}$ species in yeast) where within a family of isoacceptors a base change in the discriminator position is found (4). These results prove that a change in the 'discriminator nucleotide' does not necessarily abolish aminoacylation with the cognate amino acid.

A series of elegant studies involving chemical modification of *E. coli* $tRNA_f^{Met}$ have been done to correlate specific nucleotides in this tRNA with various biological functions of this molecule (26). These experiments showed that the anticodon, the variable loop and the acceptor stem region are affecting methionyl-tRNA synthetase recognition. Studies with tRNA modified in positions 73 or 74 suggested that these changes may lead to slower charging rates. Our finding of lowered levels of aminocylation and V_{max} values for two of the analogs suggest that *E. coli* methionyl-tRNA synthetase interacts with nucleotide 74 in the tRNA.

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