Recognition by Initiator Transfer Ribonucleic Acid of a Uridine 5′ Adjacent to the AUG Codon: Different Conformational States of Formylatable Methionine-Accepting Transfer Ribonucleic Acid at the Ribosomal Peptidyl Site†

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ABSTRACT: Initiator tRNA of *Escherichia coli* contains an unmodified adenosine 3′ adjacent to the anticodon. We examined whether during codon–anticodon interaction at the ribosome this adenosine may become implicated in recognition of a uridine 5′ adjacent to the initiation codon AUG. The triplet AUG was compared with the oligonucleotides UAUG, CAUG, and AAUG for the coding efficiency of *E. coli* initiator tRNA under various conditions. We found a uridine-specific stimulating effect of the 5′-terminal nucleotide. In the presence of initiation factors, UAUG promoted initiation complex formation with 70S ribosomes 3-fold more efficiently than did AUG, CAUG, or AAUG. However, if the fMet-$tRNA_f^{Met}$ was nonenzymatically bound to 70S ribosomes, no stimulatory effect of the 5′-terminal uridine was observed. In this case, AUG promoted initiation complex formation more efficiently than any of the tetranucleotides. With 30S ribosomal subunits, fMet-tRNA discriminates between UAUG and AUG whether or not initiation factors were present. The stimulatory effect of the 5′-terminal uridine was also observed in the absence of factors with 70S ribosomes, provided that a 30S–UAUG–fMet-tRNA complex was allowed to form prior to the addition of 50S subunits. This shows that the initiation factors are not required per se for the tetranucleotide recognition to occur but that it is the sequence of events during the 70S–UAUG–fMet-tRNA complex formation which is critical. The fMet group seems to be important for the manner in which the initiator tRNA is accommodated at the ribosome, since deacylated $tRNA_f^{Met}$ discriminates between UAUG and AUG under conditions in which the aminoacylated species does not. The results suggest that the anticodon loop of the initiator tRNA may be accommodated in different conformations at the ribosomal peptidyl site, depending on the way the tRNA enters the ribosome.

In almost all the tRNA primary sequences determined so far, the base adjacent to the 3′ end of the anticodon is a modified adenine or a modified guanine [for a collation of tRNA sequences, see Gauss et al. (1979)]. In the few cases where the base in this position is not modified, it is always adenine. In the tRNAs of *Escherichia coli* there exists a well marked correlation between the structure of the base in the third position of the anticodon and the structure of the modified base in the neighboring position [for a review, see Feldmann (1977)]. However, one interesting exception is the *E. coli* initiator tRNA, $tRNA_f^{Met}$; tRNAs with an uridine at the 3′ end of the anticodon have normally been found to contain the threonine-substituted adenosine *N*-(purin-6-ylcarbamoyl)-L-threonine ribonucleoside, t^6A, or its 6-*N*-methyl derivative, mt^6A, adjacent to the 3′ end of the anticodon, whereas in contrast *E. coli* $tRNA_f^{Met}$ contains an unmodified adenosine at this position. The functional role of hypermodification of the anticodon 3′ adjacent base has not so far been settled. It has been pointed out, however, that a common characteristic of all the modified bases occurring at this position is their inability to take part in Watson–Crick base pairing, and it was suggested that the modifications are necessary in order to define the reading frame in the anticodon loop (Parthasarathy et al., 1974).

The lack of an intrinsic mechanism in the ribosome which restricts codon–anticodon interaction to only three bases at a time was suggested by the finding that addition of one nucleotide in the anticodon loop of a tRNA resulted in suppression of a (+1) frameshift mRNA (Riddle & Carbon, 1973).

We were interested in investigating whether the anticodon 3′ adjacent adenosine might recognize a uridine 5′ contiguous with an AUG initiator codon during ribosomal fMet-$tRNA_f^{Met}$ binding. We therefore compared the coding efficiency for *E. coli* initiator tRNA of the initiation triplet AUG with the tetranucleotides UAUG, CAUG, and AAUG under various conditions. It is demonstrated that only the tetranucleotide UAUG promotes initiation complex formation much more efficiently than the triplet AUG. The results further suggest that at the 70S ribosomal peptidyl site the initiator tRNA may exist in alternative states which are capable of discriminating between UAUG and AUG or are not capable.

Experimental Procedures

Materials. *E. coli* tRNA, $tRNA^{Phe}$, and $tRNA_f^{Met}$ were supplied by Boehringer, Mannheim. ApU, CpA, ApA, UpA, and all ribonucleoside 5′-diphosphates and 5′-triphosphates were purchased from Pharma Waldhof, Düsseldorf. Polynucleotide phosphorylase from *Micrococcus luteus* (EC 2.7.7.8) was bought from Boehringer, Mannheim, and alkaline phosphatase (EC 3.1.3.1) was from Sigma, München. T_1 RNase (EC 3.1.4.8) and T_2 RNase were obtained from Sankyo Inc., Tokyo. [^{3}H]Methionine (sp act. 33 Ci/mmol) and [^{3}H]phenylalanine (sp act. 18 Ci/mmol) were bought from Amersham/Buchler, Braunschweig. *E. coli* strain MRE 600 was from the Public Health Laboratory Service, CAMR, Porton, U.K. Polyethylenimine TLC plates (CEL 300 PEI) were obtained from Macherey-Nagel & Co., Düren.

Preparation of Oligonucleotides. AUG was prepared by the combined use of polynucleotide phosphorylase (PNPase)[1]

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and ribonuclease T_1 as described (Mohr & Thach, 1969). AAU, CAU, and UAU were synthesized with primer-dependent PNPase and UDP, starting with ApA, CpA, or UpA as the primer (Schetters et al., 1972). The tetranucleotides AAUG, CAUG, and UAUG were prepared according to the following procedure. A reaction mixture (1 mL), containing 150 mM Tris-HCl, pH 9.5, 10 mM magnesium acetate, 7.5 mM trinucleoside diphosphate (AUU, CAU, or UAU), 20 mM GDP, 2 mg of PNPase, and 800 units of T_1 ribonuclease was incubated for 6 h at 37 °C. Following heat inactivation of the PNPase (5 min at 90 °C), alkaline phosphatase (15 μg) was added and the mixture incubated for 3 h at 37 °C. The reaction products were separated on a DEAE-cellulose column (1.5 × 15 cm) by using a 2 × 750 mL gradient containing 10–500 mM triethylammonium bicarbonate. The yield in tetranucleotides varied between 60 and 80%. Since the tetranucleotides were generally contaminated by residual T_1 ribonuclease, they were routinely subjected to paper chromatography, using *n*-propyl alcohol–concentrated ammonium hydroxide–water (55:10:35) as the solvent. Following elution with water, the tetranucleotides were further purified on Sephadex G-25. The nucleotide composition of the oligonucleotides was analyzed on polyethylenimine plates, following digestion by ribonuclease T_2 as described by Volckaert & Fiers (1977).

Preparation of Ribosomes, aa-tRNAs, and Initiation Factors. The 70S ribosomal tight couples from *E. coli* MRE 600 were prepared as described (Noll et al., 1973). The 50S and 30S ribosomal subunits were isolated by zonal centrifugation (Hindennach et al., 1971). The 30S ribosomal subunits were depleted of ribosomal protein S1 [30S–(S1)] as described (Steitz et al., 1977). The resulting 30S(–S1) ribosomes contained less than 5% S1, as judged by immunological methods. Ribosomal subunits were generally reactivated prior to use (Zamir et al., 1971). *E. coli* $tRNA_f^{Met}$ was aminoacylated with [^{3}H]methionine (sp act. 2 Ci/mmol) and formylated as described (Hershey & Thach, 1967). Initiation factors were prepared according to the procedure of Hershey et al. (1977), except that the separation step for IF-1 and IF-2 was omitted. Pure initiation factor IF-2 was kindly provided by Dr. Gualerzi, Berlin.

tRNA-Binding Assays. The initiation factor dependent binding of fMet-tRNA to 70S ribosomal tight couples was performed as follows. The incubation mixture of 50-μL total volume contained 50 mM Tris-HCl, pH 7.4, 100 mM NH_4Cl, 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.3 mM GTP, saturating amounts of IF-1 IF-2, and IF-3, 25 pmol of 70S ribosomes, 25 pmol of f[^{3}H]Met-$tRNA_f^{Met}$, and varying amounts of AUG or tetranucleotides as indicated. This system was incubated for 10 min at 37 °C, and the amount of f[^{3}H]Met-tRNA bound was measured by nitrocellulose filter assay (Nirenberg & Leder, 1964).

The initiation factor dependent binding of fMet-tRNA to 30S ribosomal subunits was performed as described above except that the reaction mixture contained 25 pmol of reactivated 30S ribosomal subunits and saturating amounts of IF-1/IF-2 only. Incubation was for 10 min at 20 °C. The reaction was terminated by diluting the incubation mixture with 3 mL of ice-cold buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM NH_4Cl, 10 mM magnesium acetate, and 10 mM 2-mercaptoethanol.

[1] Abbreviations used: IF-1, IF-2, and IF-3, initiation factors 1, 2, and 3, respectively; PNPase, polynucleotide phosphorylase; aa-tRNA, aminoacyl-tRNA; P-site, peptidyl site.

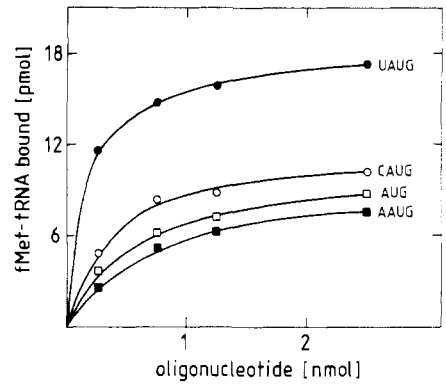

FIGURE 1: Initiation complex formation with 70S ribosomes in dependence of AUG, UAUG, CAUG, and AAUG. fMet-tRNA binding was performed in the presence of all initiation factors and the indicated amounts of initiation oligonucleotides as described under Experimental Procedures. The reaction mixtures were incubated for 10 min at 37 °C. (□) AUG; (●) UAUG; (○) CAUG; (■) AAUG.

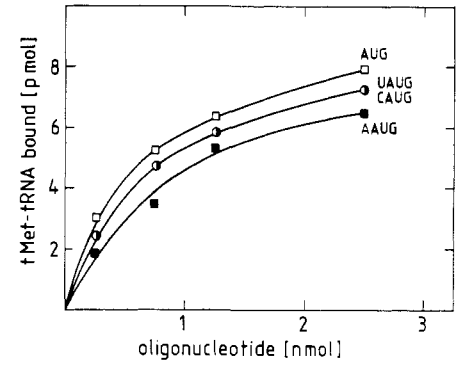

FIGURE 2: Nonenzymatic fMet-tRNA binding to 70S ribosomes with AUG, UAUG, CAUG, and AAUG. The binding of fMet-tRNA to 70S ribosomes was carried out at 10 mM Mg^{2+} in the absence of initiation factors (Experimental Procedures). Incubation was for 10 min at 37 °C. (□) AUG; (■) AAUG; (◑) UAUG or CAUG.

The nonenzymatic binding of fMet-tRNA to 70S ribosomes and 30S ribosomal subunits was performed according to the procedure described above, except that initiation factors were omitted from the reaction mixtures and the Mg^{2+} concentration was raised to 10 mM. The mixtures were incubated for 10 min at 37 °C (with 70S ribosomes) or at 20 °C (with 30S subunits).

Each binding experiment was performed at least 4 times; the scatter did not exceed 5%.

Results

Coding Properties of the Initiation Oligonucleotides with 70S Ribosomes. The initiation factor dependent binding of fMet-tRNA to 70S ribosomes was promoted quite differently by UAUG and AUG (Figure 1). At a codon concentration of 2 μM (8-fold excess over ribosomes), the initiation complex formation was 3-fold higher with UAUG as compared with AUG (Figure 1). To determine whether this highly stimulating effect of the 5′-terminal nucleotide was base specific, we investigated the tetranucleotides CAUG and AAUG; GAUG was omitted because of preparative problems. Both oligonucleotides displayed a coding behavior similar to that of AUG and not similar to that of UAUG. While CAUG was 10–15% more efficient than AUG in promoting the initiation complex formation, AAUG was even inferior to the triplet (Figure 1).

The stimulating effect of the 5′-terminal uridine was abolished, however, if the fMet-tRNA binding to 70S ribosomes was performed in the absence of initiation factors at

Table I: fMet-tRNA Binding to 70S Ribosomes in the Presence of AUG, UAUG, and Initiation Factor IF-2[a]

amount of oligonucleotide added (nmol)	fMet-tRNA bound (pmol)	
	AUG	UAUG
0.2	0.6	0.9
0.7	1.0	1.7
1.5	1.2	2.0
2.5	1.6	2.6

[a] fMet-tRNA binding to 70S ribosomes was performed as described in the legend to Figure 1, except that initiation factor IF-2 alone was added.

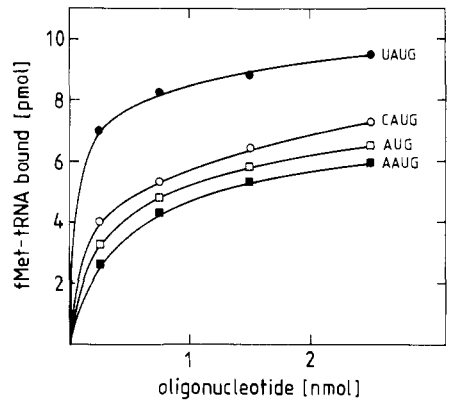

FIGURE 3: Initiation factor mediated binding of fMet-tRNA to 30S ribosomal subunits with AUG, UAUG, CAUG, and AAUG. fMet-tRNA binding to 30S subunits in the presence of saturating amounts of IF-1/IF-2 and the indicated amounts of initiation oligonucleotides was performed as described under Experimental Procedures. Incubation was for 10 min at 20 °C.

10 mM Mg^{2+}. In this system UAUG was even slightly less active than AUG and its coding behavior was indistinguishable from that of CAUG (Figure 2). A purine at the 5′ end of the starting triplet (AAUG) reduced the yield of fMet-tRNA binding more significantly under these conditions.

In order to exclude the possibility that the different coding properties of UAUG observed in the presence and absence of initiation factors are due simply to different Mg^{2+} concentrations used in the test systems, we have repeated the enzymatic fMet-tRNA binding under the same ionic conditions as the nonenzymatic binding. Again UAUG promoted initiation complex formation much more efficiently than the other oligonucleotides. The dependence of fMet-tRNA binding upon the concentration of the various oligonucleotides was virtually the same as that shown in Figure 1.

Two possibilities (not necessarily mutually exclusive) have to be considered to explain the requirement for initiation factors: (1) IF-2 might promote the quadruplet recognition between the anticodon loop and UAUG by complex formation with the initiator tRNA; (2) the tetranucleotide recognition might occur preferentially on 30S ribosomal subunits which are produced from 70S tight couples in the presence of initiation factors but not under the nonenzymatic conditions.

In the presence of IF-2 alone, UAUG promoted fMet-tRNA binding more efficiently than AUG, indicating that recognition of UAUG as a tetranucleotide was possible (Table I). The effect, however, was not very pronounced; in particular, the yields of fMet-tRNA binding were rather low. Thus the possibility cannot be excluded that the stimulating effect of UAUG under these conditions has to be ascribed to the dissociation activity of residual amounts of IF-3 present on the ribosomes. To avoid these uncertainties, we investigated the coding properties of the oligonucleotides with regard to

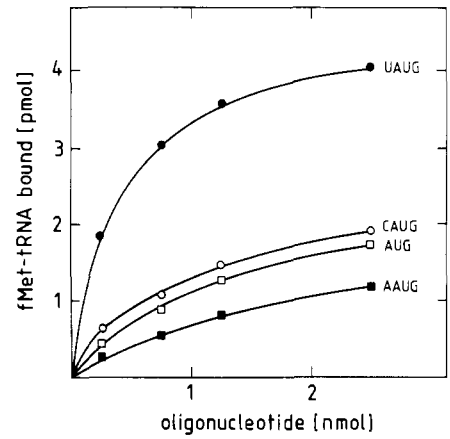

FIGURE 4: Binding of fMet-tRNA to 30S subunits with AUG, UAUG, CAUG, and AAUG in the absence of initiation factors. Nonenzymatic fMet-tRNA binding to 30S subunits was carried out at 10 mM Mg^{2+} as outlined under Experimental Procedures. Incubation was for 10 min at 20 °C.

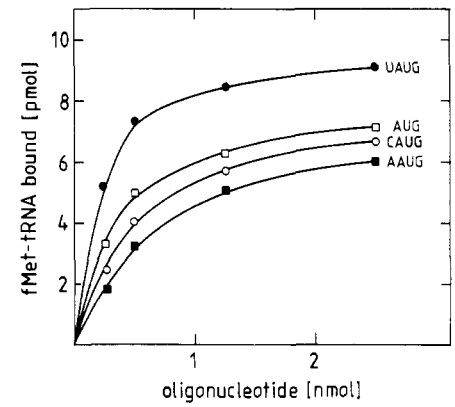

FIGURE 5: Nonenzymatic fMet-tRNA binding to 70S ribosomes with AUG, UAUG, CAUG, and AAUG, generated via an intermediate 30S–oligonucleotide–fMet-tRNA complex. In a preincubation step nonenzymatic binding of fMet-tRNA to 30S subunits dependent on the initiation oligonucleotides was performed essentially as described under Experimental Procedures. After a 10-min incubation at 20 °C, the reaction mixtures were kept on ice for 3 min. Following addition of 50 pmol of reactivated 50S subunits (2-fold excess over 30S subunits) in 5 μL containing the same buffer concentrations as the 50-μL reaction mixture, 70S complex formation was allowed for another 5 min at 0 °C. The incubation mixtures were diluted with 3 mL of ice-cold wash buffer, and fMet-tRNA binding was determined by the Millipore filter assay.

fMet-tRNA binding to ribosomal subunits.

Coding Behavior of the Oligonucleotides with Ribosomal Subunits. In contrast to the results with 70S ribosomes, the stimularoty effect of a 5′-terminal uridine on the binding of fMet-tRNA to 30S subunits was observed whether initiation factors were present or not (Figures 3 and 4). Interestingly, the relative differences in the coding efficiency among UAUG, AUG, and AAUG were even more pronounced in the absence of factors. The effect of the 5′ nucleotide was found to be uridine specific under either condition (Figures 3 and 4). This demonstrates that there is no absolute requirement for initiation factors for fMet-tRNA to discriminate between UAUG and AUG. Consequently, this should also be possible under nonenzymatic conditions with 70S ribosomes, provided that formation of the 30S–UAUG–fMet-tRNA intermediate complex is allowed.

Following preincubation of isolated 30S subunits, initiation oligonucleotide, and fMet-tRNA at 10 mM Mg^{2+}, a 2-fold molar excess of 50S subunits over small subunits was added in order to incorporate all 30S particles in 70S ribosomes.

Table II: Puromycin Reactivity of Initiator tRNA in 70S-UAUG-fMet-tRNA Complexes[a]

	initiation complexes	fMet-tRNA bound/25 pmol of 70S ribosomes (pmol)	(aminoacyl)-puromycin extracted	
			pmol	% from bound fMet-tRNA
expt A	70S tight couples; initiation factors; 5 mM Mg^{2+}, UAUG, fMet-tRNA	15	14	93
expt B	70S tight couples; 10 mM Mg^{2+}, UAUG, fMet-tRNA	7	6.5	93
expt C	30S subunits; 10 mM Mg^{2+}, UAUG, fMet-tRNA; after 10 min at 20 °C addition of 50S subunits	8	7.6	94

[a] The various 70S-UAUG-fMet-tRNA complexes were prepared essentially as described in the legends to Figure 1 (experiment A), Figure 2 (experiment B), and Figure 5 (experiment C). In each experiment the reaction mixtures contained 3 nmol of UAUG. Puromycin reactivity of fMet-tRNA was determined by adding puromycin to the incubation mixtures to a final concentration of 0.5 mM. Incubation was continued for 15 min at 0 °C. After extraction with 2 mL of ethyl acetate, 1.5 mL of the organic phase was counted according to the procedures of Leder & Bursztyn (1966).

Under these conditions, UAUG was found to be superior to the other oligonucleotides in promoting fMet-tRNA binding to 70S ribosomes (Figure 5). The entire initiator tRNA bound was found to be puromycin reactive (see below), which proves that only 70S–UAUG–fMet-tRNA complexes were measured.

If the 30S and 50S subunits were allowed to form 70S ribosomes prior to the addition of initiator tRNA, the stimularoty effect of the 5′-terminal uridine was again abolished. In this case UAUG promoted fMet-tRNA binding to the same extent as the triplet AUG (data not shown). This suggests that it is the sequence of events during the 70S–UAUG–fMet-tRNA complex formation which is critical for the recognition of UAUG, either as a tetra- or a trinucleotide, by the initiator tRNA.

Puromycin Reactivity of 70S–UAUG–fMet-tRNA Complexes Formed under Various Conditions. The fact that under some conditions (Figures 1 and 5) the binding of fMet-tRNA to the ribosome is enhanced by the 5′-terminal uridine, whereas under other conditions (Figure 2) it is not, suggests that different conformations of the complex 70S–UAUG–fMet-tRNA are produced by different experimental conditions. To determine whether these differences were related to occupation of distinct ribosomal sites, we compared the sensitivity toward puromycin of fMet-tRNA in 70S–UAUG–fMet-tRNA complexes which were formed (1) enzymatically with all initiation factors and 70S tight couples, (2) nonenzymatically with 70S tight couples, or (3) nonenzymatically with 30S–UAUG–fMet-tRNA complexes and subsequent addition of 50S ribosomal subunits. No differences in the puromycin reactivity of the fMet-tRNA bound under the various conditions were observed (Table II). In each complex more than 90% of the fMet-tRNA bound was puromycin reactive. This shows that

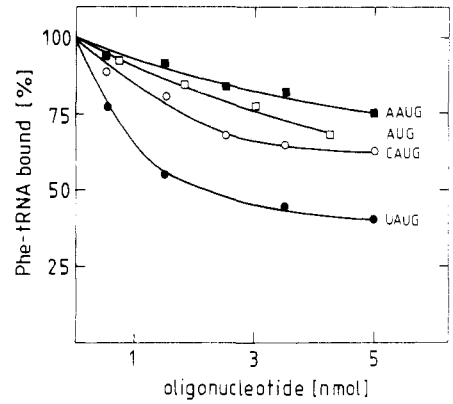

FIGURE 6: Nonenzymatic binding of deacylated $tRNA_f^{Met}$ to the P-site of 70S ribosomes with AUG, UAUG, CAUG, or AAUG, as followed by the inhibition of poly(U)-directed Phe-tRNA binding. Seventy-five microliters of a mixture containing 50 mM Tris-HCl, pH 7.4, 60 mM NH_4Cl, 8 mM magnesium acetate, 10 mM 2-mercaptoethanol, 25 pmol of 70S ribosomal tight couples, 5 μg of poly(U), 50 pmol of deacylated pure $tRNA_f^{Met}$ and the indicated amounts of initiation oligonucleotides was incubated for 5 min at 37 °C to allow oligonucleotide-dependent $tRNA_f^{Met}$ binding to the ribosomes. The sample was then supplemented with 25 μL of a mixture containing the same salt concentration as above, together with 100 pmol of EF-Tu, 0.5 mM GTP, and 25 pmol of [^{3}H]Phe-$tRNA^{Phe}$. Incubation was done for 5 min at 37 °C. The amount of [^{3}H]Phe-tRNA bound was measured by the nitrocellulose filter assay. One-hundred percent Phe-tRNA binding corresponds to 17 pmol of Phe-tRNA bound/25 pmol of 70S ribosomes.

under all conditions the initiator tRNA was bound to the ribosomal P-site.

Influence of the fMet Group on the Decoding of UAUG by $tRNA_f^{Met}$ at 70S Ribosomes. It is known that the peptidyl moiety has a strong influence on the binding of tRNA to ribosomes (Cannon et al., 1973). Accordingly, we investigated whether deacylated $tRNA_f^{Met}$ behaved differently from the aminoacylated form in decoding UAUG on 70S ribosomes. The nonenzymatic binding of deacylated $tRNA_f^{Met}$ to 70S ribosomal tight couples was followed indirectly. We measured the inhibition of the poly(U)-directed enzymatic Phe-tRNA binding by $tRNA_f^{Met}$ as a function of increasing amounts of initiation oligonucleotides. In the presence of a cognate AUG codon, $tRNA_f^{Met}$ binds to the P-site of 70S ribosomes and thus blocks any subsequent poly(U)-dependent interaction of the Phe-tRNA with the same ribosome (R. Lührmann, unpublished experiments).

Among the oligonucleotides tested, UAUG clearly caused the strongest inhibition of the poly(U)-directed Phe-tRNA binding (Figure 6). Thus, in contrast to the aminoacylated fMet-tRNA, which does not discriminate between UAUG and AUG under nonenzymatic conditions with 70S tight couples (Figure 2), the deacylated species does discriminate when entering the 70S P-site directly.

Ribosomal Protein S1 Does Not Influence the Tetranucleotide Recognition. Protein S1 has been shown to be required for mRNA binding and for initiation complex formation in response to natural mRNA (Szer et al., 1975; Van Dieijen et al., 1975). Furthermore, its location has been identified as being near to the ribosomal decoding sites (Fiser et al., 1975; Pongs et al., 1975; Lührmann et al., 1976). We compared the UAUG-dependent nonenzymatic binding of fMet-tRNA to S1-depleted 30S subunits with native 30S subunits. UAUG stimulated fMet-tRNA binding to the same extent, whether or not S1 was present on the ribosome. The dependence upon the concentration of UAUG was virtually the same as shown in Figure 4. The same lack of dependence on S1 was also observed for the UAUG-promoted initiation complex forma-

tion with 70S ribosomes (data not shown). Thus S1 does not seem to be critical for discrimination by fMet-tRNA between UAUG and AUG on the ribosome.

Discussion

In this study we show that nucleotides 5′ adjacent to the initiation codon AUG have a considerable influence on the ribosomal decoding of *E. coli* fMet-tRNA. Most strikingly, the tetranucleotide UAUG promotes initiation complex formation much more efficiently than the triplet AUG (Figures 1 and 3). Our contention that this is due to the formation of a fourth base pair between the adenosine (A37) 3′ adjacent to the anticodon of the initiator tRNA and the 5′-terminal uridine of UAUG is supported by the following findings. (1) The highly stimularoty effect of the 5′-terminal nucleotide was shown to be base specific and to be restricted to uridine. This excludes the possibility that simple addition of a phosphodiester bond on the 5′ side of AUG electrostatically enhances fMet-tRNA binding. (2) Increased base stacking in the tetranucleotide UAUG as compared to AUG can also be ruled out as a reason for the high coding activity of UAUG. AAUG, which displays stronger stacking interactions than UAUG, is inferior even to AUG in promoting fMet-tRNA binding (Figure 1). (3) The possibility that the formation of the binary ribosome–initiation oligonucleotide complex determines the efficienty of fMet-tRNA binding is also unlikely. The same sequence UAUG may be recognized by fMet-tRNA as a tri- or tetranucleotide, depending on the way the tRNA enters the ribosome (Figures 1–3). (4) Structural alterations in the 30S ribosomal mRNA binding sites would not be expected to affect greatly a tetranucleotide codon–anticodon interaction. In fact, the removal of S1 has no effect at all.

The data on the UAUG-dependent binding of fMet-tRNA to ribosomal subunits clearly show that there is no absolute requirement for initiation factors to allow the four base pair codon–anticodon interaction, suggesting that it is an inherent property of the tRNA structure. The presence of ribosomes, however, seems to be necessary for the quadruplet interaction to occur. In the absence of ribosomes, the molar association constants of $tRNA_f^{Met}$ with AUG and with UAUG are almost identical, showing that the anticodon 3′ adjacent adenosine is not available for base pairing when the tRNA is free in solution (Uhlenbeck et al., 1970).

The ability of fMet-tRNA to decode UAUG either as a trinucleotide or as a tetranucleotide indicates that the initiator tRNA may be accommodated on the 70S ribosome in different conformational states. The nature of these conformational differences is not yet known. However, the notion that they are due to tRNA occupation of different ribosomal sites can be excluded. In the various 70S–UAUG–fMet-tRNA complexes which differ in exhibiting tri- or tetranucleotide codon–anticodon interaction, the fMet-tRNA is equally reactive to puromycin (Table II). According to this criterion the tRNAs are bound to the P-site. In the case where the tetranucleotide interaction is allowed, the anticodon loop may be assumed to be in a 3′-stacked conformation similar to that found in the crystal structure of yeast $tRNA^{Phe}$ (Robertus et al., 1974; Kim et al., 1974). The restriction to the triplet codon–anticodon interaction between fMet-tRNA and UAUG under nonenzymatic conditions with 70S tight couples (Figure 2) could be explained by a 5′-stacked structure of the anticodon loop or by a minor change in the configuration around position A37, producing incompatibility with double-strand formation.

Recently, evidence was given for a unique anticodon loop conformation of initiator tRNAs in solution (Wrede et al., 1979). In several initiator tRNAs, the S1 nuclease cleavage pattern of loop II was identical and differed from that of elongation tRNAs. Most interesting is the lack of cleavage of the phosphodiester bond between the nucleoside in the third position of the anticodon and the purine in position 37 of initiator tRNAs. It would be intriguing to know whether the different conformational states adopted by the initiator tRNA at the ribosome reflect a transition between the two conformations observed for initiator and elongation tRNAs in solution.

The way in which the aminoacyl end of the tRNA interacts with the ribosome seems to be important for determining which conformation the initiator tRNA adopts on the ribosome. The conformation related to the tetranucleotide recognition is easily adopted if no interaction between the peptidyl moiety (fMet group) and the peptidyltransferase center is possible. This is the case with deacylated $tRNA_f^{Met}$ in 70S ribosomes (Figure 2) and with aminoacylated tRNA in 30S subunits (Figures 3 and 4). If binding of aminoacylated initiator tRNA to 70S ribosomes is measured without being channelled through intermediate 30S complexes (Figure 2), the initial contact of the tRNA with the ribosome may involve the fMet group and the peptidyltransferase center. This seems to cause a tRNA conformation corresponding to a trinucleotide interaction with UAUG (Figure 2).

The observed differences between the accommodation of fMet-tRNA and the deacylated species at the P-site could also be of functional significance for a chain elongation tRNA in the posttranslocational complex. Following transpeptidation, the deacylated tRNA remaining in the P-site could undergo a transition to a state which might be more favorable for leaving the ribosome during the next translocation step.

Our results agree only partially with a report in which a battery of oligonucleotides, including UAUG, CAUG, and AAUG, was used to investigate the effect of sequences at either the 5′ or 3′ side of AUG on the initiation complex formation (Ganoza et al., 1978). Under nonenzymatic conditions, AUG was found superior to all other nucleotides in promoting fMet-tRNA binding to 70S ribosomes. A purine on the 5′ side of AUG decreased the coding efficiency more strongly than a pyrimidine. This agrees with our results as shown in Figure 2. In contrast to our findings the authors claim, however, that no differences were observed between the coding properties of the oligonucleotides whether initiation factors were present or not. Unfortunately they did not investigate initiation complex formation with ribosomal subunits. Since we have shown that the sequence of events during the 70S–UAUG–fMet-tRNA complex formation is critical for the four-nucleotide codon–anticodon interaction to occur, they may have missed the specific effect of the 5′-terminal uridine on ribosomal fMet-tRNA binding. Furthermore, the interpretation of their experiments is made more problematic by the fact that only a small population of the ribosomes (less than 1%) was active in fMet-tRNA binding.

Initiation complex formation is influenced not only by nucleotides 5′ adjacent to the initiation codon but also by nucleotides contiguous with AUG on the 3′ side. Recently it was reported that in mutants of Qβ RNA, an adenosine 3′ adjacent to the initiator codon of the coat protein cistron, considerably stimulated the efficiency of 70S initiation complex formation (Taniguchi & Weissmann, 1978). The authors suggested that this might be due either to an increase in stacking interactions or to formation of a fourth base pair with U33 of the anticodon loop of fMet-tRNA. In good agreement with this contention, it was demonstrated that the tetranucleotide AUGA stimulated binding of fMet-tRNA to ribosomes at a considerably lower

concentration than AUG (Manderschied et al., 1978). A forthcoming paper reports that the 3′-terminal adenosine affects fMet-tRNA binding by influencing the stacking interactions rather than by base pairing with the tRNA (Schmitt et al., 1981). In this context it is interesting to note that the characteristics of the AUGA–fMet-tRNA interaction differ from those of the UAUG-dependent complex formation. The stimulatory effect of the 3′ adjacent adenosine was only observed in the presence of IF-2 for the 30S initiation complex formation. With 70S ribosomes AUGA was inferior to AUG. It would be interesting to examine whether the pentanucleotide UAUGA acts as a "super initiator codon" or whether it functions in the same way as one or another of the constituent tetranucleotides UAUG or AUGA.

In our study we have attempted to generate base-pair formation between the anticodon 3′ adjacent adenosine of the initiator tRNA and the 5′-terminal uridine of UAUG and to exploit this to detect conformational differences in the tRNA on the ribosome. Since the results show a stimulating effect of the 5′-terminal uridine on fMet-tRNA binding under conditions most suitable for initiation of translation, it is reasonable to assume that a similarly placed uridylic acid residue in natural mRNA could provide a similar enhancement of initiation complex formation.

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