INITIATOR-tRNA RECOGNIZES A TETRANUCLEOTIDE CODON DURING THE 30 S INITIATION COMPLEX FORMATION

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1. Introduction

The recognition mechanism whereby the correct AUG initiation codon of a mRNA binds either to the 30 S ribosome or to the 30 S × IF-2 × fMet-tRNA$_f^{Met}$ complex has not been elucidated as yet [1]. Aside from codon–anticodon double strand formation, there is definitely some stabilizing interaction between the intercistronic sequence within a polycistronic mRNA on the one hand and the 3′-end of the 16 S RNA and the ribosomal protein S1 on the other hand [2]. In most monocistronic eukaryotic mRNAs the so-called cap m^{7}G$^{5'}$ pppN may bind to the 40 S ribosome [3].

Two recent articles, have shed some light on the molecular mechanism involved in the formation of a correct initiation complex.

(i) In the case of Qβ RNA an effective initiation complex is formed only with the AUGG and AUGA in the coat protein cistron but not with a Qβ RNA having a mutant initiator codon AUAG [4]. Half initiation activity, however, was regained when point mutations leading to AUAA occurred.

(ii) By using ALMV-RNA and AcPhe-tRNA instead of fMet-tRNA the tetranucleotide UUUA preceeding the AUG by about 10 codons is recognized as an initiator [5]. AcVal-tRNA, however, did not recognize the neighboring sequence GUUU.

These data prompted us to investigate the possibility that during 30 S initiation complex formation the initiator-tRNA does not bind to a triplet but to a tetranucleotide, preferentially to AUGG or AUGA. In the following, we present experimental evidence to confirm this assumption and discuss the role of the constant U_{33} next to the anticodon in initiation complex formation.

Abbreviations: GMPPCP, guanylyl-methylendiphosphonate; AcVal-tRNA, Val-tRNA acylated at the αNH_2 group

2. Experimental

2.2. *Materials*

Escherichia coli tRNA^{Phe}, tRNA^{Lys}, tRNA$_f^{Met}$, and nucleoside diphosphates were purchased from Boehringer, Mannheim. [^{3}H]Phenylalanine, [^{3}H]methionine, [^{3}H]valine and [^{3}H]lysine were obtained from Amersham-Buchler, Braunschweig and *Escherichia coli* MRE 600 type from Merck, Darmstadt.

2.2. *Preparation of components*

70 S ribosomes were prepared from mid log *E. coli* according to [6] and further purified to yield 70 S 'tight couples' by the method in [7]. 30 S ribosomal subunits were isolated from these tight couples by zonal centrifugation [8]. They were activated before use, as in [9]. tRNAs were aminoacylated with [^{3}H]phenylalanine, [^{3}H]valine, or [^{3}H]lysine as [10]. tRNA$_f^{Met}$ was aminoacylated with [^{3}H]methionine and formylated following the method in [11] but omitting the deacylation step. [^{3}H]Val-tRNA^{Val} was *N*-acetylated according to the method in [12]. Two-fold recrystallized elongation factor EF-T$_u$ was prepared as in [13]. Initiation factors were obtained by the procedure in [14] except that in an additional step IF-2 was concentrated by

adsorption to a phosphocellulose column. AUG was prepared according to [15] and was elongated with nucleoside diphosphates using primer-dependent polynucleotide phosphorylase (EC 2.7.7.8.) to yield $AUGN_n$ [16].

3. Results

The oligonucleotide-directed and factor-dependent binding of either fMet-tRNA or aa-tRNA to 30 S or 70 S ribosomes was measured by the nitrocellulose filter assay.

The AUG- and AUGA-dependent binding of the initiator-tRNA to 30 S and 70 S ribosomes is shown in fig.1. With 30 S ribosomes AUGA stimulates fMet-tRNA binding at a considerably lower concentration than AUG and AUGU while with 70 S ribosomes AUGA is inferior to AUG. For the elongator-tRNA, Val-tRNA, the reversed situation is observed (fig.2). GUAA does not, in contrast to GUA, stimulate Val-tRNA binding to 30 S ribosomes, while the extent

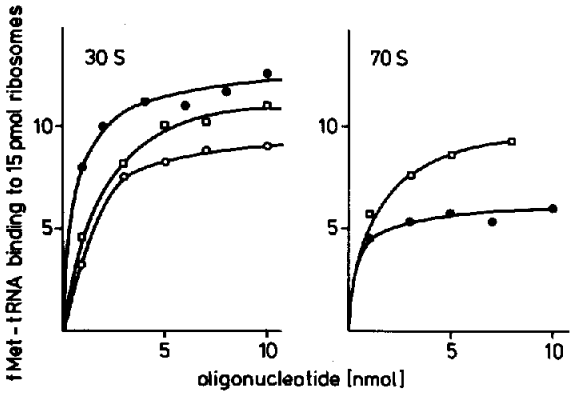

Fig.1. AUG, AUGU, and AUGA dependent f[^{3}H]Met-tRNA binding to 30 S and 70 S ribosomes. The incubation mixture contained in final vol. 50 μl: 50 mM Tris–HCl, pH 7.4, 100 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 1 mM dithioerythrol, 15 pmol 70 S ribosomes, saturating amounts (10 μg) of IF-2, 35 μg IF-1/IF-3, 20 pmol f[^{3}H]Met-tRNA *E. coli* (spec. act. 1000 Ci/mol), and varying amounts of oligonucleotides. With 30 S subunits the mixtures contained 15 mM $Mg(OAc)_2$, 20 μg IF-2, and 15 pmol 30 S ribosomes, otherwise the mix was the same as before. The samples were incubated at 20°C for 10 min, and the amount of f[^{3}H]Met-tRNA bound was measured by nitrocellulose filter assay. Blanks without oligonucleotide were subtracted. AUGA (●——●); AUG (□——□); AUGU (○——○).

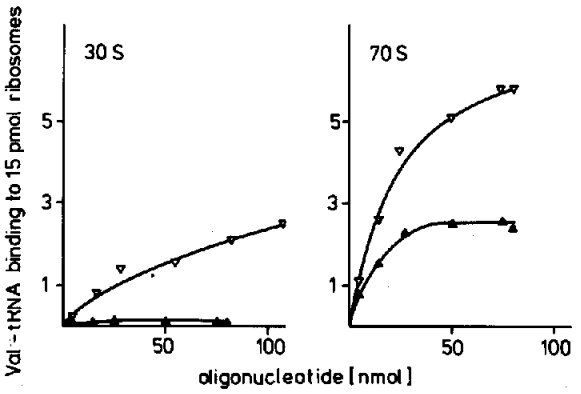

Fig.2. GUA- and GUAA-dependent [^{3}H]Val-tRNA binding to 30 S and 70 S ribosomes. Experimental conditions were as described in the legend to fig.1, except that the $Mg(OAc)_2$ concentration was 10 mM, and incubation was carried out at 0°C for 40 min. GUA (▽——▽); GUAA (▲——▲).

of binding to the 70 S ribosomes in response to GUAA is comparable to the response to GUA in the 30 S system. These findings are in agreement with those reported earlier for Phe-tRNA (UUC/UUCA) and Lys-tRNA (A_3/A_4) binding to 30 S ribosomes [19].

Since for fMet-tRNA binding to 30 S ribosomes at

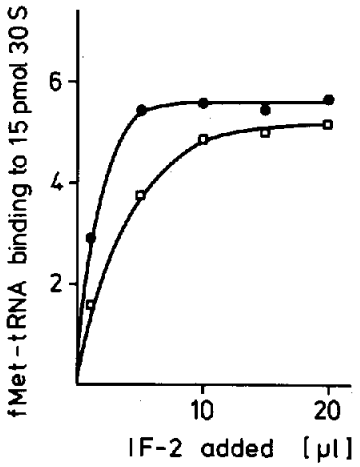

Fig.3. IF-2 dependence of f[^{3}H]Met-tRNA binding to 30 S ribosomes at half-saturating concentrations of oligonucleotide. The reaction mixtures were the same as in fig.1 legend, but contained constant amounts of oligonucleotide (3 nmol AUG or AUGA) and varying amounts of IF-2 (1 μg/μl). Blanks without IF-2 were subtracted. AUG (□——□); AUGA (●——●).

low Mg^{2+} concentration, stoichiometric amounts of IF-2 · GTP are required we investigated the role of IF-2 in AUG and AUGA mediated binding of fMet-tRNA to 30 S ribosomes. Figure 3 shows for the latter that the concentration of IF-2 required to reach saturation is much lower, while the degree of saturation is independent of the type of oligonucleotide used. The influence of IF2 is even more pronounced in case of AcVal-tRNA binding to 30 S ribosomes (fig.4). In the absence of IF-2 this pseudo-initiator-tRNA does not bind in response to GUAA while in its presence the GUAA concentration required to saturate the binding is very low. Qualitatively the same observations are made for GUA-mediated binding, although the influence of IF-2 is less striking. Thus IF-2 GTP seems to hold the tRNA in a conformation capable of tetranucleotide codon–anticodon interaction.

The observation that 30 S ribosomes are unable to bind two aa-tRNAs simultaneously in response to a hexanucleotide was difficult to rationalize up to now [1]. In the present context, however, the interpretation is obvious and we therefore reinvestigated the $AUGU_3$ mediated binding of Phe-tRNA to 30 S and 70 S ribosomes in the presence and absence of fMet-tRNA · IF-2 · GTP. Figure 5 demonstrates the inhibition of Phe-tRNA binding to 30 S ribosomes by this

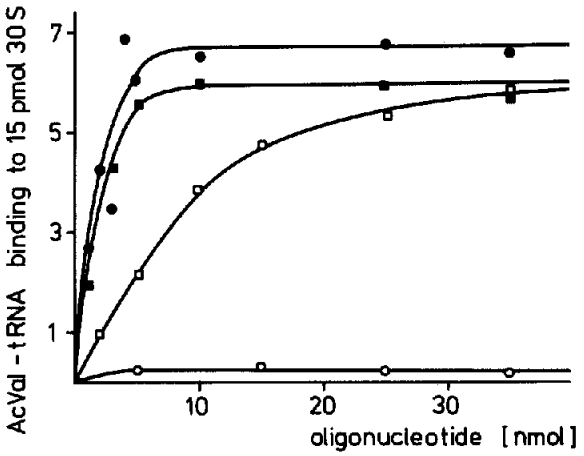

Fig.4. GUA- and GUAA-dependent Ac[^{3}H]Val-tRNA binding to 30 S and 70 S in the presence or absence of initiation factors. Experimental conditions were as described in the legend to fig.1. Minus initiation factors (open symbols); plus initiation factors (closed symbols); GUAA (circles); GUA (squares).

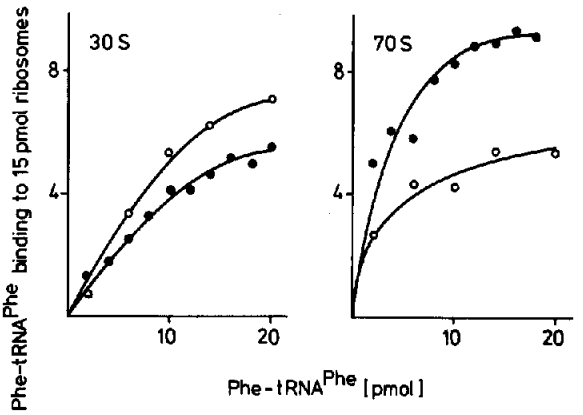

Fig.5. $AUGU_3$ dependent [^{3}H]Phe-tRNA^{Phe} binding to 30 S and 70 S ribosomes in the presence or absence of fMet-tRNA$_f^{Met}$ · IF-2 · GTP. The reactions were carried out in two steps. In the first step (compare legend to fig.1), fMet-tRNA$_f^{Met}$ · IF-2 · GTP were allowed to react with the ribosomes. Samples without the ternary complex were incubated under identical conditions. In a second step 50 µl of an elongation mixture containing 100 mM NH_4Cl, 50 mM Tris–HCl pH 7.4, 1 mM DTE, 10 mM GTP, 1 µl EF-Tu (10 mg/ml), 25 mM $Mg(OAc)_2$ with 70 S (15 mM when 30 S were used), and varying amounts of [^{3}H]Phe-tRNA^{Phe} yeast, spec. act. 5000 Ci/mol, were added to each sample. Incubation was continued at 0°C for 40 min. Plus fMet-tRNA$_f^{Met}$ · IF-2 · GTP (●——●); minus fMet-tRNA$_f^{Met}$ · IF-2 · GTP (○——○).

complex, while the same ternary complex increased the extend of Phe-tRNA binding to the 70 S ribosome. Here naturally GTP is hydrolyzed and IF-2 is removed from the complex. U_6 as a control showed the binding of 6 pmol Phe-tRNA to both 15 pmol 30 S and 70 S ribosomes (data not shown). $AUGA_3$ was not used for these experiment, since Lys-tRNA binding is only in the range of 1–2 pmol for both 30 S and 70 S ribosomes. The reasons for that are not understood at the moment.

4. Discussion

Studying the known mRNA sequences one finds that in monocistronic mRNA the initiation triplet is followed by a codon starting with G or A [17]. In the case of polycistronic mRNA the situation is more complex. The 30 S · fMet-tRNA$_f^{Met}$ · IF-2 · GTP

complex binds mainly to the coat protein initiator region, where the codon is either AUGG or AUGA [1]. At cistrons like the A-protein or the replicase, where initiation occurs to a lower extent, codons starting with pyrimidine nucleotides can be found following the AUG. It appears to us that the preferential initiation at the coat protein cistron is not the result of the tertiary structure of the mRNA but is due to the higher affinity of the AUGA/G tetranucleotide for the initiator tRNA as compared to AUGU of AUGC [4]. Thus the base sequence of the initiation tetranucleotide regulates the preferential binding of the 30 S · fMet-tRNA$_f^{Met}$ · IF-2 · GTP to a certain cistron.

Tetranucleotides terminating in adenosine exhibit a 10-fold higher association constant for the elongator-tRNA and the initiator-tRNA in ribosome-free systems as compared to the ones terminating in a pyrimidine nucleoside [18]. The striking effect is, that the tetranucleotides are inactive in stimulating the binding of an elongator-tRNA to the 30 S [19], whereas with the initiator-tRNA, AUGA is more effective than AUG and AUGU. The dominant role of IF-2 can be derived from fig.3 where AcVal-tRNA binding to 30 S ribosomes is stimulated by GUAA in the presence of IF-2.

If one accepts the tetranucleotide double strand formation as a prerequisite for a stable 30 S initiation complex, then the addition of the 50 S subunit, GTP hydrolysis, and subsequent IF-2 dissociation has to break the fourth base interaction before the aminoacyl site in the 70 S initiation complex is open for the binding of the ternary complex aa-tRNA · EF-T_u · GTP. This explains an earlier observation that IF-2 inhibits the positioning of the aa-tRNA to the A-site in the 70 S initiation complex and that aa-tRNA binding is blocked if GTP is replaced by GMPPCP [20]. The switch from the tetranucleotide recognition on the 30 S to a triplet interaction on the 70 S ribosome requires a conformational change of the initiator-tRNA tertiary structure, which has to result in a withdrawal of the constant U_{33} from interaction with the mRNA. Whether this 'interaction' is a Watson Crick type base pairing or is a stacking type interaction as assumed [21] cannot be decided here.

Support for the assumption that a positional flexibility of U_{33} is possible was provided [22] in that he was able to show partial availability of the nucleotide to chemical modification. Since CGAA interaction with the TψCG sequence in the elongator-tRNA prevents the tetranucleotide interaction in codon anticodon recognition, a similar mechanism may play a role in the 70 S initiation complex formation [23].

In summary our data support a tetranucleotide interaction between fMet-tRNA$_f^{Met}$ · IF-2 · GTP · 30 S complex and the initiator codon of the cistron to be translated. Aminoacyl-tRNA binding to the 30 S initiation complex is blocked due to the overlap interaction with the first nucleotide of the elongator codon. Binding of the aa-tRNA · EF-T_u · GTP complex cannot take place prior to the removal of IF-2 from the 70 S initiation complex.

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