POLYURIDYLIC ACID-DEPENDENT BINDING OF fMet-tRNA TO *ESCHERICHIA COLI* RIBOSOMES AND INCORPORATION OF FORMYLMETHIONINE INTO POLYPHENYLALANINE

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1. Introduction

It is generally accepted that initiation of protein synthesis in *Escherichia coli* starts with *N*-formylmethionine, directed by the codons AUG or GUG. In one case, reinitiation on the mRNA of amber mutants of *lac* repressor, UUG is used as the initiation codon [1].

Early studies [2,3] indeed showed that the triplets AUG, GUG and UUG are the most effective in stimulating fMet-tRNA binding to ribosomes in vitro.

Synthetic polynucleotides containing AUG and/or GUG codons as well as natural mRNA have been used extensively in order to elucidate the mechanism of initiation of protein synthesis (reviewed in [4]).

In all these studies it has been assumed that binding of fMet-tRNA to ribosomes is directed by AUG or GUG codons present in the polynucleotide.

This paper describes a study in which poly(U) is used to direct fMet-tRNA binding to 30 S ribosomes. The fMet is incorporated into polyphenylalanine when 50 S subunits and other factors for protein synthesis are added.

2. Materials and methods

2.1. *Ribosomal subunits and initiation factors*

Ribosomes were isolated from *E. coli* strain MRE 600 and were washed in standard buffer (10 mM Tris–HCl (pH 7.6); 10 mM $Mg(OAc)_2$; 60 mM NH_4Cl; 6 mM β-mercaptoethanol) containing 1 M NH_4Cl to yield crude initiation factors. Purified initiation factors were prepared by the method in [9]. All factors were pure according to analysis on SDS–gel electrophoresis.

30 S and 50 S subunits were isolated from 70 S ribosomes by dissociation in standard buffer at 1 mM $Mg(OAc)_2$ and sucrose gradient centrifugation and reactivated 30 min at 37°C in standard buffer at 20 mM $Mg(OAc)_2$ before storage at −80°C.

2.2. *fMet-tRNA, MS2 RNA, poly(U), (A), (X), (C), (I) and (U)14 and UUU*

f[^{3}H]Met-tRNA (5300 cpm/pmol) was prepared as in [10]. Bacteriophage MS2 was grown in *E. coli* Q13. Purification and extraction of the RNA was performed as in [10].

Poly(U), poly(A), poly(C) and UUU were obtained from Boehringer Mannheim. Concentrations were determined from measurements of the ultraviolet absorbancy [11].

Poly(X) and poly(I) were obtained from P.-L. Biochemicals. Concentrations of poly(X) and poly(I) were obtained from measurements of the ultraviolet absorbancy (poly(I): $E_{max} = 10.2 \times 10^3$ at 248 nm; poly(X): $E_{max} = 7.8 \times 10^3$ at 249 nm).

(U)14 was a gift of Drs R. Lipecky and H. G. Gassen [12].

2.3. *Binding of fMet-tRNA to 30 S subunits*

Assay mixtures with 50 μl final vol. contained: 40 mM Tris–HCl (pH 7.6); 40 mM NH_4Cl, 6 mM $Mg(OAc)_2$; 6 mM β-mercaptoethanol; 0.2 mM GTP, 20 pmol f[^{3}H]Met-tRNA and 0.2 A_{260} 30 S ribosomal subunits.

The amounts of MS2 RNA, poly(N) and initiation

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factors are as indicated in the figure and table legends. The mixtures were incubated 5 min at 37°C, diluted 50-fold with ice-cold wash-buffer (20 mM Tris–HCl (pH 7.6); 60 mM NH_4Cl; 6 mM $Mg(OAc)_2$; 6 mM β-mercaptoethanol) and filtered on nitrocellulose filters (Selectron BA-85).

2.4. *T_1 RNase digestion*

Poly(U), 10 A_{260}, and MS2 RNA were digested with 10 units T_1 RNase [8] in a buffer containing 100 mM Tris–HCl (pH 7.6) and 10 mM $Mg(OAc)_2$ for 20 min at 37°C. T_1 RNase was removed by 3-times phenol extraction in the presence of 1% SDS. Phenol was removed from the water layer by 3-times extraction with ether.

The RNAs were precipitated with 2 vol. ethanol. Control samples of MS2 RNA and poly(U) were treated in the same way with the omission of T_1 RNase.

2.5. *Amino acid incorporation*

fMet-tRNA was incubated with 30 S ribosomes as mentioned before. 50 S subunits, 0.6 A_{260}, were added and the mixture was incubated for 20 min at 37°C. Then all components for translation (5 nmol [^{14}C]phenylalanine (82 cpm/pmol) 50 μg supernatant fraction, energy, pyruvate kinase and 30 μg tRNA) were added and further incubated for 20 min at 37°C at 8 mM $Mg(OAc)_2$ followed by the addition of 1 ml 5% trichloroacetic acid. After 10 min at 90°C the reaction mixture was chilled and filtered over GF/A filters.

3. Results

Figure 1 shows that poly(U) stimulates the binding of fMet-tRNA to 30 S ribosomes. The efficiency of binding is comparable to a phage (MS2) RNA programmed system, both with respect to mRNA concentration and to ribosome concentration (data not shown).

The binding of fMet-tRNA to ribosomes, either programmed by AUG triplet, poly(A,U,G) or natural mRNA is strictly dependent on IF-2 [5,6]. This is also the case here (fig.2). IF-2, in the absence of other factors, is absolutely required. In contrast, IF-1 can be omitted, but its addition stimulates fMet-tRNA binding 2–3-fold (fig.3). IF-3 is not required and in fact inhibits the binding (fig.4). This is different from systems programmed with MS2 RNA where a strong requirement for IF-3 is found [6].

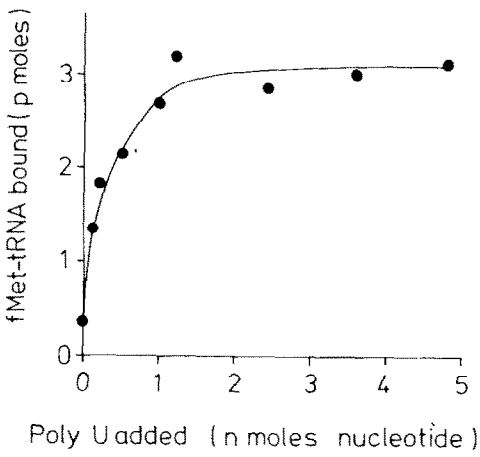

Fig.1. Binding of fMet-tRNA to 30 S ribosomes as a function of the poly(U) input. Assay mixtures contained 0.85 μg IF-2 and 0.08 μg IF-1.

The inhibitory effect of IF-3 on fMet-tRNA binding to MS2 RNA programmed 30 S ribosomes is also observed when the factor is added beyond saturating amounts [7].

Several polynucleotides were tested for their ability to stimulate the binding of fMet-tRNA to 30 S ribosomes

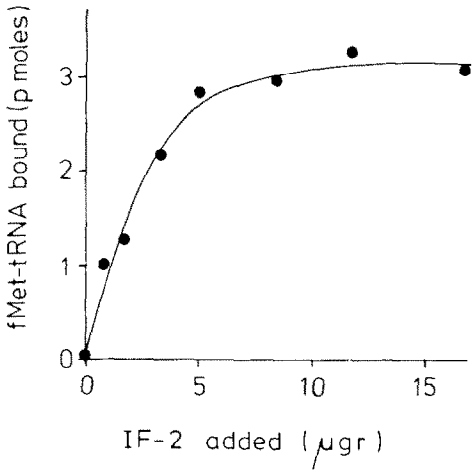

Fig.2. Binding of fMet-tRNA to 30 S ribosomes as a function of the IF-2 input. Assay mixtures contained 3 nmol poly(U).

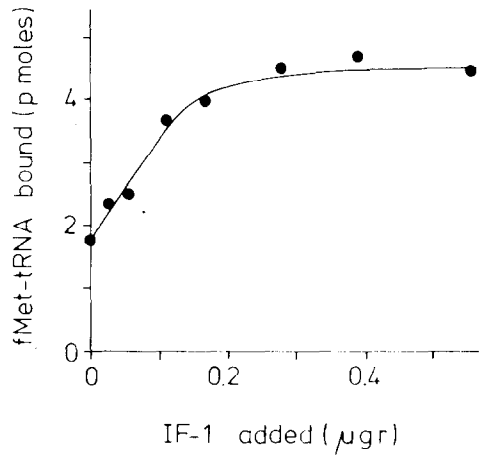

Fig.3. Binding of fMet-tRNA to 30 S ribosomes as a function of the IF-1 input. Assay mixtures contained 3 nmol poly(U) and 1.7 μg IF-2.

(table 1). Only poly(U) and poly(X) were active in this respect. Poly(C), poly(A) and poly(I) gave no stimulation above the background and (U)14 and (U)3 were also not active.

fMet-tRNA bound to 30 S ribosomes in the presence of poly(U) is released as fMet-puromycin when 50 S subunits and puromycin are added (table 2).

Furthermore, fMet is incorporated into hot-acid

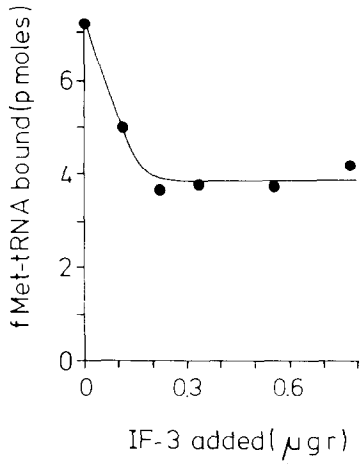

Fig.4. Binding of fMet-tRNA to 30 S ribosomes as a function of the IF-3 input. Assay mixtures contained 3 nmol poly(U), 1.7 μg IF-2 and 0.11 μg IF-1.

Table 1
Binding of fMet-tRNA to 30 S subunits with different synthetic poly- and oligonucleotides

Template	fMet-tRNA bound
Poly(U)	4.20
(U)14	0.51
(U)3	0.62
Poly(C)	0.44
Poly(A)	0.64
Poly(X)	3.90
Poly(I)	0.77
None	0.56

Assay mixtures contained: 1.3 μg IF-2; 0.11 μg IF-1 and 3 nmol nucleotide

precipitable polypeptide when the complex of fMet-tRNA and 30 S is incubated with 50 S subunits, energy, supernatant fraction and phenylalanine (fig.5).

Since it has been shown that except AUG and GUG, UUG stimulates binding of fMet-tRNA to ribosomes in vitro [2,3] and is used as initiation codon in vivo [1] it was of crucial importance to rule out the presence of UUG codons in poly(U) to account for fMet-tRNA binding and fMet incorporation. In the experiment of fig.1 the maximal level of 3 pmol fMet-tRNA bound is reached at a poly(U) input of about 1 nmol U residues and hence a 0.3% contamination of poly(U) with G residues would introduce an amount of UUG which is equimolar with the fMet-tRNA bound.

However, the following experiment rules out the possibility that UUG is responsible for fMet-tRNA

Table 2
Formation of fMet-puromycin from bound 30 S fMet-tRNA

Components	fMet-tRNA bound (pmol)	fMet-puromycin (pmol)
30 S	3.4	0.27
50 S	–	0.20
30 S + 50 S	–	3.2

Assay mixtures contained 3 nmol poly(U), 0.11 μg IF-1, 1.3 μg IF-2 and 50 μg puromycin. In the last experiment binding of fMet-tRNA to the 30 S ribosomes was followed by addition of 0.6 A_{260} 50 S. After a further incubation of 5 min at 37°C, 0.5 ml 0.4 M Na(OAc) pH 5.5 was added and fMet puromycin was extracted with ethylacetate

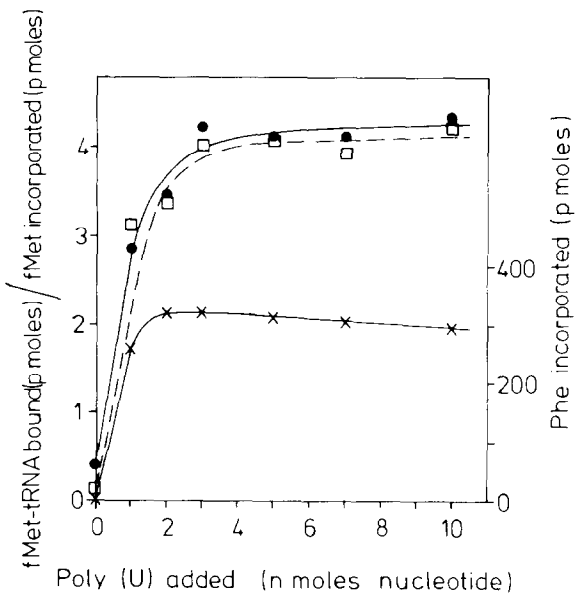

Fig.5. Binding of fMet-tRNA to 30 S ribosomes and incorporation of fMet into polyphenylalanine. Assay mixtures contained 0.11 μg IF-1 and 1.3 μg IF-2. (–●–●–●–) fMet-tRNA bound to 30 S subunits; (–□–□–□–) fMet incorporated in polypeptides; (–×–×–×–×–) Phe incorporated in polypeptides.

binding and fMet incorporation in our experiments: Poly(U) was treated with ribonuclease T_1 at conditions where MS2 RNA was completely degraded into oligonucleotides [8]. Control samples of poly(U) and MS2 RNA were treated similarly in the absence of the ribonuclease. Control and T_1-digested poly(U) and MS2 RNA were reisolated and tested for their ability to stimulate the incorporation of fMet in a ribosomal system. The results are shown in table 3. While fMet and Ala incorporations are completely abolished when MS2 RNA is treated with T_1, treatment of poly(U) with this nuclease has little effect.

If binding and incorporation of fMet had been due to the presence of UUG, treatment with T_1 RNase would have positioned this codon at the ends of the polynucleotide chains and hence it could not code for N-terminal fMet. This experiment, of course, also excludes the presence of AUG and GUG as initiation codons in poly(U).

4. Discussion

Our data show that poly(U) is capable of stimulating the binding of fMet-tRNA to 30 S ribosomes. This binding is 'functional' in so far that the fMet is incorporated (N-terminal) into polyphenylalanine upon addition of 50 S ribosomes, energy, supernatant enzymes and phenylalanine.

The possibility that the binding of fMet-tRNA to the 30 S ribosome is due to the presence of AUG, GUG or UUG triplets in poly(U) has been excluded. Rigourous treatment of poly(U) with ribonuclease T_1 which should split the polynucleotide chain after each G residue neither results in significant degradation of poly(U) (data not shown), nor abolishes fMet-tRNA binding and fMet incorporation (table 3). Since neither UUU triplet nor (U)14 stimulates fMet-tRNA binding,

Table 3
Effect of T_1 RNase treatment on the incorporation of fMet in polypeptide

	Template added (nmol nucleotide)	T_1 RNase treatment	fMet-tRNA incorp. (pmol)	Phe/Ala incorp. (pmol)
A.	Poly(U) 17	yes	1.87	141
	Poly(U) 15	no	2.08	216
	–	–	0.07	1.8
B.	MS2 RNA 78	yes	0.05	0.34
	MS2 RNA 54	no	0.49	9.52
	–	–	0.06	0.40

Assay mixtures contained: (A) 0.11 μg IF-1, 1.3 μg IF-2 and 5 nmol [^{14}C]phenylalanine; (B) 0.11 μg IF-1, 1.3 μg IF-2, 0.2 μg IF-3, 1 nmol of each amino acid and [^{14}C]alanine (290 cpm/pmol)

we conclude that simple interaction of poly(U) with 30 S ribosomes, triggers the latter to bind initiator tRNA.

Of all the polynucleotides tested (table 1) only poly(U) and poly(X) stimulate fMet-tRNA binding to 30 S ribosomes. These polynucleotides resemble one another in the 6-membered ring structure of the base having 2 keto-groups flanking an imino-group.

The relationship between the ability of poly(U) to trigger fMet-tRNA binding to 30 S ribosomes and the process of natural polypeptide chain initiation is unclear at the moment and is a matter for further investigations.

The general assumption, however, that fMet-tRNA binding to ribosomes is correlated with the presence of an initiator codon is not supported by our experiments.

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