THE STIMULATION OF LABELLED POLYNUCLEOTIDE BINDING TO KREBS II ASCITES AND *ESCHERICHIA COLI* RIBOSOMES BY DEACYLATED tRNAs.

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1. Introduction

The sequence of nucleotide base triplets in messenger RNA determines the order in which amino acid specific tRNAs are bound to the ribosome and hence the amino acid sequence of the protein. In contrast, initiation requires the binding of messenger RNA to an initiation complex, comprising Met-$tRNA_f^{Met}$, initiation factors and small ribosomal subunits. Thus in initiation the normal order of events is reversed [1–3]. The selection and correct phasing of the initiation codons of natural messenger RNA is assured by the use of the special initiator tRNA, Met-$tRNA_f^{Met}$. A pseudo- or primitive initiation pathway, which is utilized frequently during in vitro studies of the elongation function of the ribosome, has been shown by Culp et al. [4] to involve deacylated tRNAs. The work described in this paper was undertaken to investigate whether in this primitive initiation process the anticodons of a number of deacylated tRNAs are able to direct binding of various labelled synthetic homopolynucleotides to 70S and 80S ribosomes.

2. Materials and methods

^{3}H-labelled poly (U), poly (A) and poly (C) were purchased from Schwarz-Mann (Orangeburg, N. Y., USA) and were diluted with the corresponding unlabelled polynucleotides from Sigma (St. Louis, Missouri, USA) to a spec. act. of approx. 3 Ci/mol. Details are given in the appropriate table legends. In all cases the synthetic polynucleotides sedimented well ahead of a phenylalanyl-tRNA, indicating a sedimentation constant significantly larger than 4S. $tRNA^{Gly}$ (10 pmol/μl), $tRNA^{Glu}$ (species II, 10 pmol/μl), $tRNA^{Lys}$ (10 pmol/μl), $tRNA_f^{Met}$ (10 pmol/μl), $tRNA^{Val}$ (10 pmol/μl), $tRNA^{Tyr}$ (10 pmol/μl) and $tRNA^{Phe}$ (10 pmol/μl) from *E. coli* MRE 600 were purchased from Boehringer (Mannheim, Germany). $tRNA^{Arg}$ (10 pmol/μl) and $tRNA^{Leu}$ (species I, 10 pmol/μl) were purchased from Miles Laboratories (Kankakee, Illinois, USA) and were from *E. coli* K-12 MO. $tRNA^{Ser}$ (species I and II, 10 pmol/μl) from brewer's yeast was a generous gift of Dr H. G. Zachau, Munich, and $tRNA^{Ile}$ (10 pmol/μl) from *E. coli* D 10 was kindly supplied by Dr F. M. Unger of this institute. Phenylalanine-tRNA was charged with [^{14}C]phenylalanine of spec. act. 78 Ci/mol (from the Radiochemical Centre, Amersham, England), according to published methods [5]. The isolation of $tRNA_f^{Met}$ (ascites) was also previously described [5]. Methods for the preparation of elongation factor 1 [6] and 2 [7] have been described. *E. coli* 'run-off' ribosomes were washed three times with 0.5 M NH_4Cl. Ascites 'run-off' ribosomes were freed from EF-2 contamination by two incubation steps with 1 mM GTP in the presence of creatine phosphate and creatine kinase for 15 min at 30°C and centrifugation in the presence of 0.5 M NH_4Cl. 40S and 60S ascites ribosomes subunits were prepared as described [8].

2.1. *Polynucleotide binding assay*

The incubation mixtures contained the following components in a final vol of 0.1 ml: 0.05 M KCl, 0.02 M NH_4Cl, 0.03 M Tris–HCl (pH 7.9 at 20°C),

Table 1
Conditions for the binding of [^{3}H]polyuridylic acid to ribosomes and ribosomal subunits from Krebs II ascites tumor cells

Additions				[^{3}H]poly (U) bound	[^{14}C]poly-tRNA bound
40S	60S	80S	^{14}C Phe-tRNA	(nmol nucleotide)	(pmol)
–	–	+	–	2.4	–
–	–	+	+	7.2	1.4
+	–	–	+	1.0	1.0
–	+	–	+	1.6	0.7
+	+	–	+	7.2	1.0

Binding assays were carried out as described in materials and methods. Where indicated 53 pmol [^{14}C]Phe-tRNA^{Phe} were present.

0.02 M sucrose, 0.006 M Mg $(CH_3COO)_2$, 0.004 M dithioerythritol, 0.2 mM guanosine-5′-P_2-CH_2-P or GTP, 320 μg of ascites ribosomes or 210 μg 60S and 110 μg 40S subunits or 260 μg of *E. coli* D 10 ribosomes, 30 μg of either tritiated poly (U), poly (A) or poly (C) and the indicated species and amounts of tRNAs. The reactions were started with the addition of ribosomes and lasted for 30 min at 30°C.

2.2. *Sedimentation analysis in sucrose density gradients*

In order to estimate the amount of ribosome-bound polynucleotide, the incubation mixture described above was diluted to a final volume of 0.2 ml with a buffer of the same salt concentration as in the reaction mixture. The samples were layered on 5–16% linear sucrose gradients of 17 ml prepared in buffer containing 0.08 M KCl, 0.03 M Tris–HCl (pH 7.9) and 0.006 M Mg $(CH_3COO)_2$ and centrifuged at 27 000 rev/min using the Spinco SW 27 rotor at 4°C. A four-hour centrifugation period was used for the analysis of ascites ribosomes, whereas a five hour centrifugation was found more suitable for *E. coli* ribosomes. The gradients were then pumped through an Uvicord II ultraviolet scanner and recorder and 0.9 ml fractions were collected into counting vials. The fractions were diluted with 10 ml of Instagel (Packard) and counted in a Packard 3385 liquid scintillation counter. The amount of radioactivity associated with the ribosome peak was expressed as nanomoles of ribosome-bound nucleotide.

3. Results

3.1. *Conditions which permitted polynucleotide binding to ascites ribosomes*

The initial experiments carried out with labelled poly (U) showed that neither ribosomal subunit alone was able to direct the binding of poly (U) in the presence or absence of Phe-tRNA. However, when the two subunits were combined a clearcut binding was observed as shown in table 1. Under the conditions in which a significant amount of [^{3}H]poly (U) was attached to ribosomes, only a negligible quantity of ^{14}C-labelled phenylalanyl tRNA was bound. This lack of quantitative correlation between bound [^{14}C]Phe-tRNA and [^{3}H]poly (U) suggested that it was the deacylated proportion of the tRNA preparation which had promoted [^{3}H]poly (U) binding. Therefore, in a second experiment tRNA^{Phe} and [^{14}C]Phe-tRNA^{Phe} were compared with respect to their capacity to stimulate the binding of [^{3}H] poly (U) to ribosomes (fig.1).

As indicated in panel A of fig.1, the presence of EF-1 and guanosine-5′-P_2-CH_2-P permitted significant binding of [^{14}C]Phe-tRNA^{Phe} in the ribosomal A-site. Simultaneously, [^{3}H]poly (U) was also bound. Panel B indicates that an identical amount of [^{3}H]poly (U) is bound to the ribosomes when EF-1 is omitted from the experiment described in panel A. Panel C further indicates that the amount of poly (U) bound is still the same when deacylated tRNA^{Phe} is used instead of

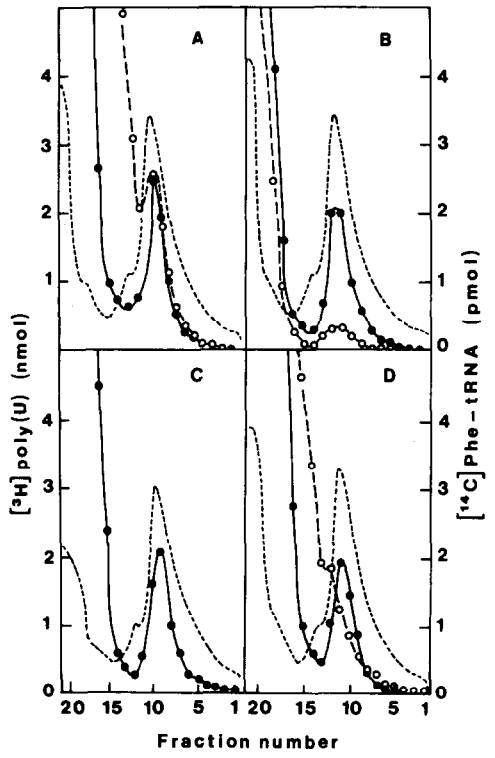

Fig.1. The influence of EF-1, EF-2 and deacylated $tRNA^{Phe}$ on the binding of [^{3}H]poly (U) to 80S ribosomes. Each tube contained ascites cell ribosomes, [^{3}H]poly (U) and guanosine 5′-P_2-CH_2-P as specified above. In addition the following components were introduced: (A) 20 μg purified EF-1 (100 pmol) and 53 pmol [^{14}C]Phe-$tRNA^{Phe}$ (173 dis. min^{-1} $pmol^{-1}$). (B) As in A, but no EF-1. (C) 40 pmol of deacylated $tRNA^{Phe}$. The experiment depicted in panel D was carried out in two steps: 9 μg EF-2 (80 pmol) were incubated with ribosomes, guanosine 5′-P_2-CH_2-P and [^{3}H]poly (U) for 10 min. EF-1 and [^{14}C]Phe-$tRNA^{Phe}$ were then added for the remaining 20 min of incubation. The samples were analyzed by sucrose density centrifugation. Dotted lines: relative absorbance at 260 nm. Solid line and closed circles: [^{3}H]poly (U). Dashed line, open circles: [^{14}C]Phe-$tRNA^{Phe}$. Direction of sedimentation left to right.

the Phe-$tRNA^{Phe}$ as in the previous two diagrams. It was, therefore, concluded that P-site occupation by deacylated $tRNA^{Phe}$ determines the capacity of the ribosome to bind poly (U).

EF-1 and EF-2 are bound to 80S ribosomes under the conditions described in panels A and D respectively [7]. It can, therefore, be concluded that the presence of elongation factors on the ribosomes does not interfere with the binding of the homopolynucleotide.

In order to examine whether the observed enhancement of poly (U) binding is anticodon-specific, dependent upon $tRNA^{Phe}$, as opposed to other tRNAs, homopolynucleotide bonding studies were performed in the presence of a number of different pure amino-acid specific tRNAs. These studies were performed with three polynucleotides, poly (U), poly (A), and poly (C) and with ribosomes from both *E. coli* and ascites Krebs II cells.

It had to be ensured that the tRNA concentrations in the following experiments were adequate. Therefore, the binding of [^{3}H]poly (U) to ascites ribosomes in the presence of increasing amounts of $tRNA^{Phe}$ and $tRNA_f^{Met}$ was studied. The results of these experiments are shown in fig.2. Saturation of polynucleotide binding was reached with approx. 200 pmol of either tRNA. Therefore, the experiments which follow were carried out with 250 pmol of each tRNA tested, which is in excess of the saturation level.

3.2. *The influence of a number of deacylated tRNAs on the binding of three different polynucleotides to 70S and 80S ribosomes*

The data from the experiments involving the

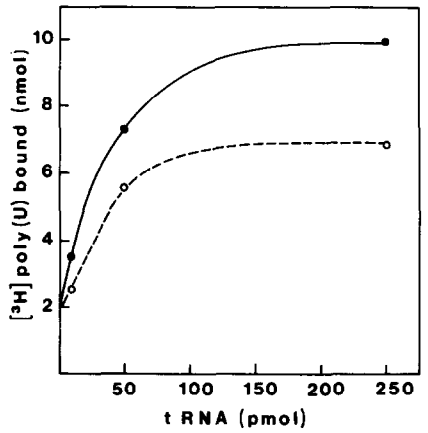

Fig.2. Binding of [^{3}H]poly (U) to 80S ribosomes in the presence of increasing concentrations of *E. coli* $tRNA^{Phe}$ and $tRNA_f^{Met}$ respectively. Binding assays and sucrose density gradient centrifugations were carried out as described for fig.1. Dashed line: binding of [^{3}H]poly (U) stimulated by $tRNA_f^{Met}$. Solid line: binding of [^{3}H]poly (U) in the presence of $tRNA^{Phe}$.

Table 2
The influence of deacylated tRNAs on the binding of tritiated poly (U) and poly (A) to 70S and 80S ribosomes.

tRNA Type	Source	Labelled polynucleotide bound (nmol) 70S poly (U)	70S poly (A)	80S poly (U)	80S poly (A)
–	–	3.5	1.7	2.4	1.1
$tRNA^{Gly}$	*E. coli*	3.6	2.4	2.1	1.2
$tRNA^{Glu}_{II}$	*E. coli*	3.7	1.4	**4.2**	1.6
$tRNA^{Lys}$	*E. coli*	3.5	**7.8**	2.8	**5.4**
$tRNA^{Met}_{f}$	*E. coli*	3.6	1.8	**7.4**	0.6
$tRNA^{Met}_{m}$	ascites	–	–	**6.0**	–
$tRNA^{Val}$	*E. coli*	3.6	2.1	**4.0**	0.7
$tRNA^{Ser}_{I+II}$	yeast	3.3	1.6	**3.8**	1.0
$tRNA^{Arg}$	*E. coli*	3.5	1.9	2.0	0.4
$tRNA^{Tyr}$	*E. coli*	3.4	1.7	2.8	1.5
$tRNA^{Ile}$	*E. coli*	3.8	1.8	**6.6**	0.8
$tRNA^{Phe}$	*E. coli*	**7.1**	1.8	**7.8**	0.1
$tRNA^{Leu}_{I}$	*E. coli*	–	–	2.9	**3.3**

Binding reactions were carried out and analyzed as described in Materials and methods. The values presented are averages from up to four seperate assays run under standard conditions and represent nmoles of nucleotide bound to either 260 μg ribosomes from *E. coli* or 320 μg ascites tumor cell ribosomes. The numbers in bold are polynucleotide binding values which are significantly higher than the tRNA-free background value.

binding of poly (U) and poly (A) to ribosomes are summarized in table 2. As can be seen, the binding of [^{3}H]poly (U) or [^{3}H]poly (A) to *E. coli* ribosomes is only stimulated by those tRNAs which bear anticodons entirely complementary to the two homopolynucleotides: $tRNA^{Phe}$ and $tRNA^{Lys}$ respectively. Other species like $tRNA^{Met}_{f}$ with unrelated anticodons did not stimulate the binding of either polynucleotide. With 70S ribosomes, this high degree of specificity was found over a wide range of Mg^{++} concentrations (6–25 mM, results not shown). In marked contrast, only partial specificity was detected with ascites ribosomes, in so far as [^{3}H]poly (U) binding was not only stimulated by $tRNA^{Phe}$ but also to the same extent by $tRNA^{Met}_{f}$ and to a lesser degree by $tRNA^{Ile}$, $tRNA^{Val}$, $tRNA^{Glu}_{II}$, $tRNA^{Ser}_{I+II}$, and $tRNA^{Tyr}$. The degree of specifictiy of ascites ribosomes was also lower in the case of poly (A) binding. Here, beside $tRNA^{Lys}$, $tRNA^{Leu}_{I}$ showed some stimulating effect. The majority of the tRNAs used in this study were derived from bacterial sources. In order to ensure that the origin of the tRNA is not influencing the specificity of polynucleotide-binding in a given system, studies were undertaken which involved the investigation of poly (U) binding to 80S ribosomes in the presence of $tRNA^{Met}_{m}$ from ascites cells. As in the case of $tRNA^{Met}_{f}$, significant poly (U) binding was observed with 80S ribosomes in the presence of this tRNA. These observations lead to the conclusion that the source of the tRNA is immaterial in deter-

mining whether 80S ribosomes exhibit absolute codon–anticodon interaction specificity or not.

Similar studies were also undertaken with [^{3}H] poly (C). Unavailability of the specific tRNA made it difficult to correlate the data obtained with those obtained from studies with the other two polynucleotides tested. Very low [^{3}H]poly (C) binding values were obtained in the absence or presence of tRNAs, suggesting that the poly (C)–ribosome interaction is relatively weak. A similar conclusion was arrived at by Takanami and Okamoto [9], who found that poly (C) was associated to only a small extent with 70S ribosomes. They attributed this weak association to the high melting temperature of poly (C). Similarly, Moore [10], showed that poly (U) could displace pre-bound poly (C) from ribosomes.

4. Discussion

It is well established that the ribosomal P-site has a strong affinity for deacylated tRNAs. During the normal peptide elongation process, after each transfer of the peptide residue to the incoming aminoacyl-tRNA, bound in the A-site, the deacylated tRNA is still fixed in the P-site and must be removed by the elongation factor EF-G (or EF-2) to allow translocation. Thus, for example, it has been shown in both bacterial [11] and higher systems [12,13] that the presence of any deacylated tRNA inhibits the initiator-tRNA binding to the P-site.

The results presented in this paper indicate that the type of deacylated tRNA bound to the ribosomal P-site strongly influences the capacity of the ribosome to bind a particular homopolynucleotide sequence. In the case of *E. coli* ribosomes, the anticipated stimulation of poly (U) or poly (A) binding by $tRNA^{Phe}$ or $tRNA^{Lys}$ was observed. The results obtained with ascites ribosomes suggested a lower binding specificity, thus, poly (U) binding could be stimulated by a number of deacylated tRNAs in addition to $tRNA^{Phe}$. The origin of this lack of specificity remains unclear at this point. Topographical information has been derived from these experiments concerning the relative location of the messenger or polynucleotide binding sites relative to the elongation factor binding sites. The experiments showed that the presence of elongation factors on the ribosome has no influence on the ribosome's capacity to bind polynucleotides, and it was, therefore, concluded that these two binding sites are distant from one another. In view of the known mechanism of protein synthesis, in which the peptide bearing tRNA is translocated from the A-site of the ribosome into the P-site after transpeptidation with concomitant movement of messenger RNA relative to the ribosome, it is not suprising that there should remain some codon–anticodon interaction also in the P-site of the ribosome. The results presented in this paper represent direct proof that such an interaction does exist and that its specificity differs in 70S and 80S ribosomes.

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