ON THE COVALENT BINDING OF mRNA MODELS TO THE PART OF THE 16 S RNA WHICH IS LOCATED IN THE mRNA BINDING SITE OF THE 30 S RIBOSOME

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Received May 15,1975

SUMMARY

The oligonucleotide U-U-|^{3}H|U-$(nh_2)^{2'}$U as a mRNA model was bound covalently to the 16 S RNA via the bifunctional guanosine-specific reagent p-chlorocarboxyphenylglyoxal. Since the ribosomes labeled with the oligonucleotide stimulated the binding of Phe-tRNA, it is concluded, that the probe reacted specifically with a guanosine of the 16 S RNA which forms the mRNA binding site of the 30 S ribosome. The binding of the probe was inhibited by poly(U), but stimulated by the presence of Phe-tRNA. 83 % of the radioactivity was found in the 16 S RNA of the ribosomal subunit. After digesting the 16 S RNA with ribonuclease T_1 and separating the oligonucleotides formed by gel electrophoresis, the radioactive labeled material was mainly found in one distinct band.

INTRODUCTION

The topography of the 30 S ribosome, namely the spatial arrangement of the 19 proteins and the 16 S RNA is at the present a field of intensive research |1-3|. Most methods i.e. crosslinking by bifunctional reagents, affinity labeling, complex formation between RNA and protein and immunological methods center on the identification of the location of the proteins with respect to each other and to the 16 S RNA.

Much less is known about the possible catalytic role of the 16 S RNA. Most chemical modification

experiments result in a loss of a certain function of the ribosome and may either be attributed to a direct involvement of the RNA or to the change in protein conformation due to an altered complex formation between RNA and protein. There is, however, good evidence, that the part of the 16 S RNA which is located within the aminoacyl-site of the 30 S ribosome plays an important role in the binding of the mRNA and in the recognition of the AA-tRNA [4-8]. Although the primary sequence of the 16 S RNA and many of its protein binding sites are known, we have up to now very little knowledge of the part of the 16 S RNA which forms the aminoacyl-site [9]. In order to identify these exposed regions of the 16 S RNA, we tried to bind oligonucleotides as mRNA models covalently to the 16 S RNA in the aminoacyl-site of the ribosome. In the following we report on the synthesis of the oligonucleotide probe, the covalent binding to the 30 S ribosome and the template activity of the oligonucleotide labeled ribosomes.

MATERIALS AND METHODS

[^{3}H]Uridine-diphosphate and [^{3}H]phenylalanine were obtained from the Radiochemical Centre, Amersham U.K., ribonuclease T_1 (EC 2.7.7.26) from Boehringer, Mannheim, and Escherichia coli MRE 600 (mid-log phase) and M. luteus cells were supplied by the Merck Company, Darmstadt. The ribosomes were isolated from E. coli cells by standard procedures [10]. They were sedimented through 35 % sucrose and were washed once with 0.5 M NH_4Cl. The 70 S ribosomes were dissociated in 10 mM Tris-HCl pH 7.2, 0.7 mM $MgCl_2$, 20 mM KCl and 0.2 mM EDTA-K; the 30 S and 50 S subunits formed were separated by zonal centrifugation [11]. The subunits were stored in activating buffer (20 mM $Mg(OAc)_2$, 200 mM NH_4Cl and 20 mM Tris-borate pH 7.2) at -80°C. Prior to use,

they were activated for 25 min at 40°C in the same buffer [12]. The synthesis of the 2'-amino-2'-deoxyuridine-5'-diphosphate ($pp(nh_2)^{2'}U$) from 2'-azido-2'-deoxyuridine [13] followed closely the procedure published by Lührmann et al [14] with reduction of the 2'-azidogroup as the last step of the reaction. The oligonucleotide U-U-[^{3}H]U-$(nh_2)^{2'}U$ (spec. act. 20 Ci/mole) was synthesized with the aid of polynucleotide phosphorylase [15]. p-Carboxylicphenylglyoxal was reacted with thionylchlorid to yield the p-chlorocarboxyphenylglyoxal. The oligonucleotide dissolved in 0.5 M phosphate buffer pH 7.8 and an ether solution of the reagent were shaken vigorously at room temperature for 10 min. Excess reagent was removed by gelfiltration on Sephadex G-25. The synthesis of the probe is depicted in Fig. 1.

RESULTS

In order to test the reactivity of the probe, it was incubated with poly(U,G) or with G-G$(-U)_7$ respectively (Fig. 2). About 600 pmoles of U-U-[^{3}H]U-

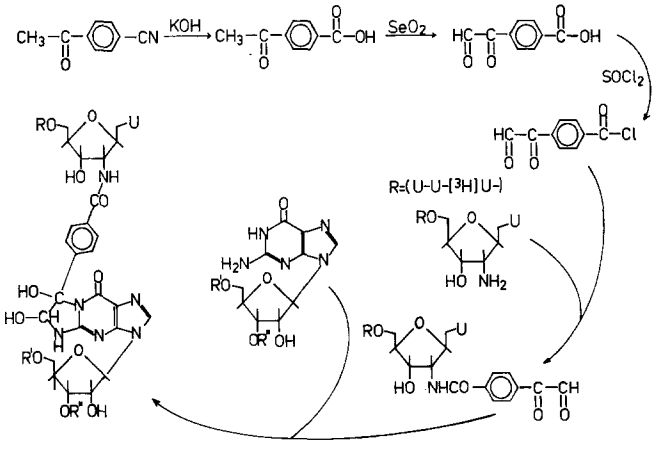

Fig. 1 CHEMICAL SYNTHESIS OF THE PROBE AND ITS BINDING TO THE GUANINE BASE OF A RNA.

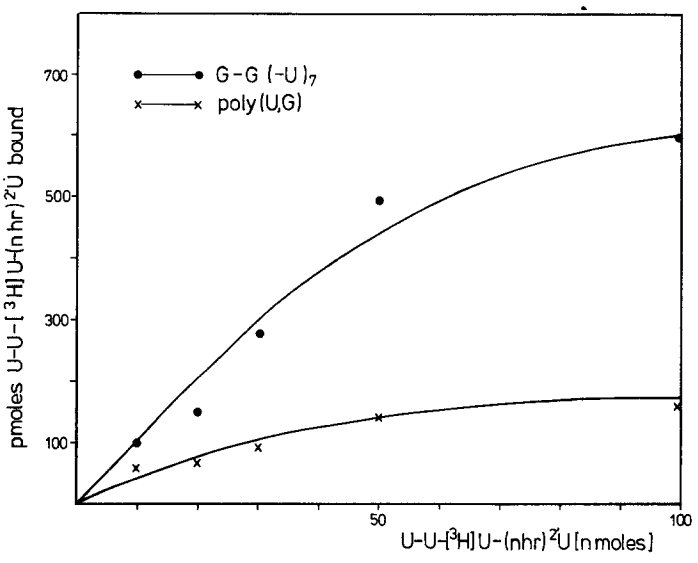

Fig. 2 BINDING OF U-U-$|^{3}H|$U-(nh)$^{2'}$U TO GUANOSINE CONTAINING OLIGONUCLEOTIDES.

The reaction mixture (20 mM Tris-borate pH 7.2) contained in a total volume of 30 µl 0.2 A_{260}-U. poly(U,G) or 0.2 A_{260}-U. G-G(-U)$_7$ and was incubated for 1 h at 37°C. The polynucleotides were separated from excess reagent by paper chromatography in 1 M NH_4OAc : C_2H_5OH 1:1.

(nhr)$^{2'}$U were bound to 2 nmoles G-G(-U)$_7$. The less efficient binding to poly(U,G) may be due to the stabile secondary structure of this polynucleotide |16|.

None of the other common nucleosides reacted to a measurable extend. The tetranucleoside triphosphate with a terminal 2'-aminouridine shows a template activity which is about 50 % lower compared to U-U-U-U, but comparable to U-U-U (Fig. 3). This may be caused by the pK-value (5.2) of the 2'-amino-group. For covalent binding the oligonucleotide was incubated with 30 S ribosomes in the presence and absence of Phe-tRNA as shown in Fig. 4. In the presence of saturating amounts of Phe-tRNA 250 pmoles of reagent were bound to 500 pmoles of 30 S ribo-

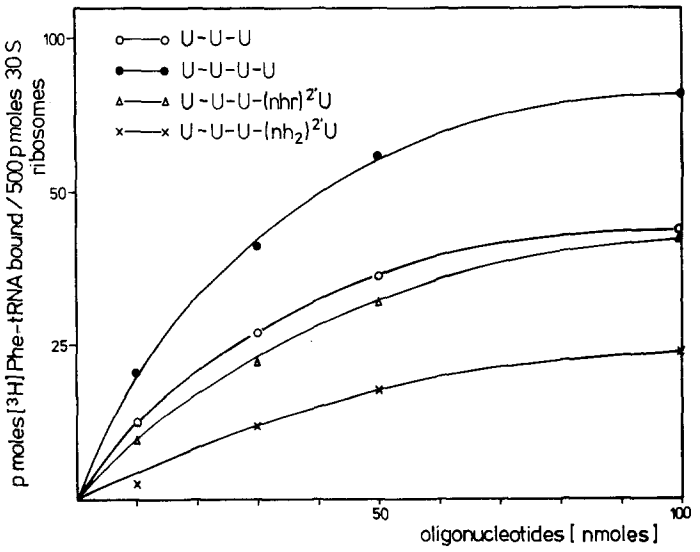

Fig. 3 STIMULATION OF $|^3H|$Phe-tRNA BINDING TO 30 S RIBOSOMES IN PRESENCE OF 3'-TERMINAL AMINOURIDINE-CONTAINING OLIGONUCLEOTIDES.

The following mixture (total volume 100 µl) was incubated for 1 h at 0°C : 10 µl 30 S ribosomes (80 A_{260}-U./ml), 30 µl $|^3H|$Phe-tRNA (40 A_{260}-U./ml, spec. activity 1000 Ci/Mol, charged 1.6 %), 50 µl mix (20 mM $Mg(OAc)_2$, 150 mM NH_4Cl, 50 mM Tris-borate pH 7.2) and the amounts of oligonucleotide indicated. Otherwise the procedure of Nirenberg and Leder was followed |18|.

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somes within 1 h at 0°C. About 40 pmoles out of 250 should be bound noncovalently by ribosome-codon-tRNA complex formation (see Fig. 3). In order to show that the oligonucleotide was bound to the mRNA binding site of the 30 S, it was tested whether the oligonucleotide-labeled ribosomes could bind Phe-tRNA. Stimulation of Phe-tRNA binding by U-U-U-$(nh_2)^{2'}$U was used as a control.

As can be seen in Fig. 5 three times the amount of Phe-tRNA was bound by U-U-U-$(nhr)^{2'}$U compared to U-U-U-$(nh_2)^{2'}$U after preincubating the 30 S ribosomes for the times indicated. With U-U-U-$(nh_2)^{2'}$U

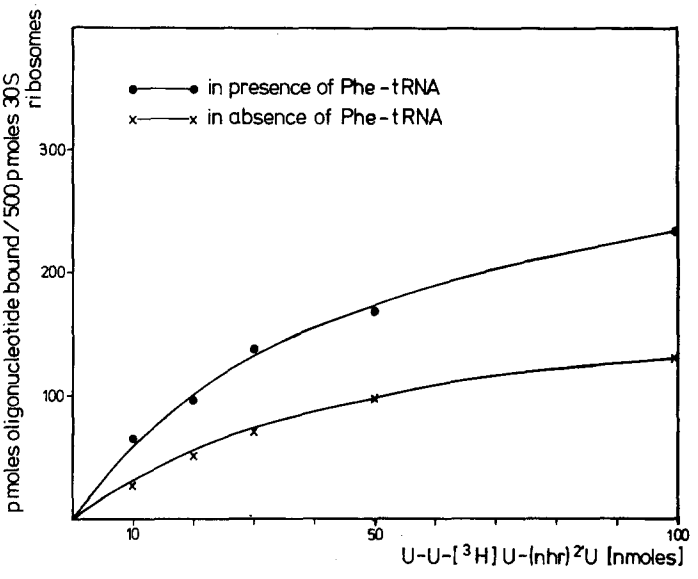

Fig. 4 COVALENT BINDING OF THE PROBE TO 30 S RIBOSOMES IN THE PRESENCE AND ABSENCE OF Phe-tRNA.

The reaction mixture was incubated for 1 h at 0°C to minimize nuclease degradation of the oligonucleotide. With the exception that |^{3}H|Phe-tRNA was replaced by Phe-tRNA the same conditions were used as discribed under Fig. 3.

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as template a plateau of binding was reached after 30 min, wherease the activity of U-U-U-(nhr)$^{2'}$U continued to increase even after 4 h.

As further evidence for the codon activity of the covalently bound oligonucleotide 30 S ribosomes were incubated with the reagent for 1 h at 0°C. The ribosomes were precipitated with 65 % ethanol and were centrifuged at 30 000xg for 20 min at -5°C. The pellet was washed with 65 % ethanol until reagent was undetectable in the supernatant. Control ribosomes (see table 1) were treated in the same manner. After suspending the ribosomes in buffer, Phe-tRNA was added and the mixture was incubated for 1 h at 0°C. The template activity of these ribosomes in shown in table 1.

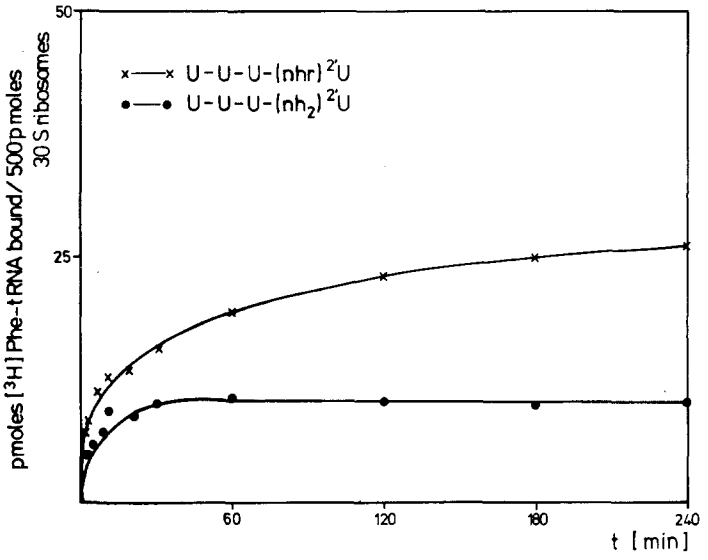

Fig. 5 STIMULATION OF $|^3H|$Phe-tRNA BINDING BY COVALENTLY BOUND U-U-U-(nhr)$^{2'}$U.

The reaction mixture was the same as discribed under Fig. 3. The total volume, however, was increased to 1 ml. 100 µl aliquots were withdrawn at the time intervals indicated and were assayed for $|^3H|$Phe-tRNA binding.

In order to show that the probe was specific for 16 S RNA and did not react with the ribosomal proteins 3 nmoles of 30 S ribosomes and 300 nmoles of reagent were incubated at 0°C for 2 h. Following precipitation with ethanol, centrifugation and washing, the ribosomes were suspended in 500 µl buffer (150 mM NaCl, 15 mM citrate-Na, 10 mM EDTA-K, 50 mM Tris-borate pH 7.2, 1 % SDS) and were extracted with phenol equilibrated with same buffer. The aqueous phase was passed over a Sephadex G-100 column (Fig. 6). Since unfractionated tRNA was used in the assay, a large proportion of the probe was bound to the tRNA. Out of 12 nmoles 4 nmoles were bound to the 30 S ribosomes, 83 % to the 16 S RNA and 17 % to the protein. The labeled 16 S RNA was hydrolysed with RNase T_1 and the oligonucleotides

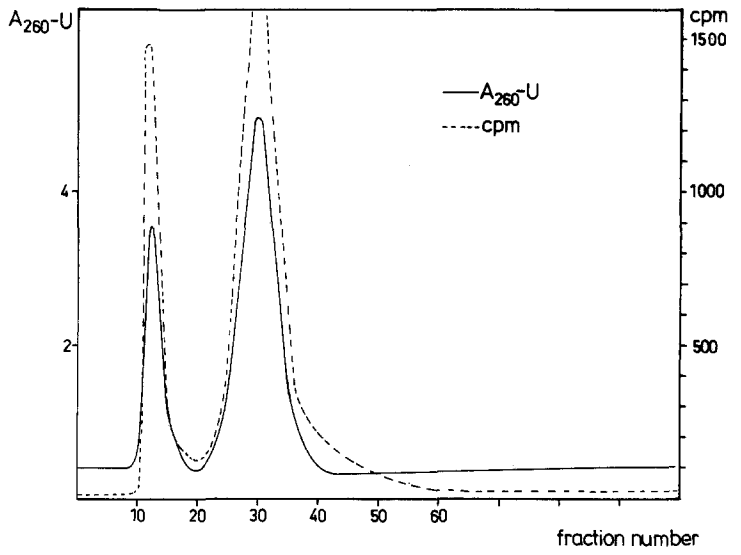

Fig. 6 SEPARATION OF LABELED 16 S RNA ON A SEPHADEX G-100 COLUMN.

The aqueous phase obtained from phenol extraction of the labeled 30 S ribosome was passed over a Sephadex G-100 column (1 cm x 50 cm). It was eluted with 50 mM Tris-borate pH 7.2 in the cold. Aliquots were assayed for radioactivity on glasfiber filters.

TABLE 1

$|^3H|$Phe-tRNA BINDING TO OLIGONUCLEOTIDE LABELED RIBOSOMES.

30 S ribosomes were incubated at $0^{\circ}C$ for 1 h in the presence of excess reagent. 50 pmoles of 30 S were removed to measure $|^3H|$Phe-tRNA binding. To control 3 saturating amounts of poly(U) were added to the 30 S ribosomes to test for remaining activity.

	labeled 30S	1 control 30S	2 control 30S	3 control 30S
probe	U-U-U-(nhr)$^{2'}$U	-	U-U-U-(nh$_2$)$^{2'}$U	-
poly(U)	-	-	-	+
$|^3H|$Phe-tRNA	+	+	+	+
$|^3H|$Phe-tRNA bound/ 50pmoles 30 S	5.4 pmoles	0.2	0.4	15.0

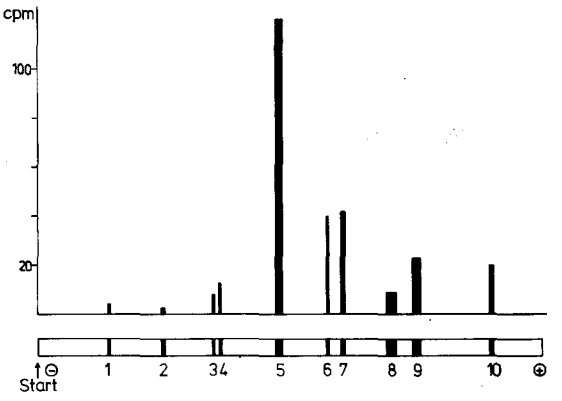

Fig. 7 SEPARATION OF OLIGONUCLEOTIDES OBTAINED FROM RNase T_1 DIGESTION OF LABELED 16 S RNA ON A 12 % POLYACRYLAMIDE GEL.

The electrophoresis was performed at 400 V, 25 mA/tube for 3 h. Following staining in methylene-blue and destaining for 12 h, the bands were sliced and counted following known procedures.

obtained were separated on a 12 % polyacrylamide gel [17]. Fig. 7 shows the distribution of the radioactivity. The main portion of the radioactivity is found in band 5.

DISCUSSION

In order to identify single stranded areas of the 16 S RNA which are located in the mRNA binding site of the 30 S ribosome, we selected a phenylglyoxal derivative, since it reacts specifically with non hydrogen-bonded guanine [19,20]. It furthermore shows the advantage of guanylic acid modification in that it becomes RNase T_1 resistant, thus making identification of the oligonucleotide possible. Since the second part of the reagent consists of an uridine-oligonucleotide it should be bound in the presence of Phe-tRNA in the first step electrostatically to the mRNA binding site of the ribosome. In a second step the glyoxylic

part of the reagent should react with the guanine moiety of the RNA.

Since the oligonucleotide still actively bound Phe-tRNA, it may be concluded that the binding occured in the close neighborhood of the mRNA binding site. According to the gel electrophoresis the oligonucleotide labeled should be located in part C of the 16 S RNA |21,22|. This would be consistent with our previous data which showed that protein S 15, forms a complex with part C of the 16 S RNA |2|, and also becomes covalently labeled by a mRNA analogue affinity label |23|.

ACKNOWLEDGEMENT

This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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