IDENTIFICATION OF A 16S RNA SEQUENCE LOCATED IN THE DECODING SITE OF 30S RIBOSOMES

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1. Introduction

It has been well established that some of the individual ribosomal proteins play a functional role in specific steps of protein biosynthesis, and detailed information about the topography of the proteins within the ribosomal subunits is known [1–4]. In addition Held et al. [5], Helser et al, [6], Noller [7] and Steitz and Jakes [8] have shown a functional involvement of 16S RNA in the 30S subunit. For example, tRNA binding to the 30S subunit could be inhibited by modification of 16S RNA with kethoxal (Noller [7]).

Recently, we were able to show [9] that the mRNA analogue U-U-U-nhr$^{2'}$U (r = –CO–C_6H_4–CO–CHO) reacts with the 16S RNA in 30S ribosomal subunits. Since the reaction was inhibited by prebinding poly (U) and Phe-tRNA^{Phe} and since the covalently linked oligonucleotide stimulated the binding of Phe-tRNA^{Phe}, it was concluded that the analogue was reacting with the 30S ribosomal decoding site [9]. We have now been able to localise the site of reaction within the primary structure of 16S RNA.

Our results clearly demonstrate that section I (about 450 nucleotides away from the 5′-end of the 16S RNA) either represents or is in the close proximity to the codon binding site of the 30S ribosome.

2. Materials and methods

2.1. *Materials*

A 250 ml culture of *Escherichia coli* MRE 600 was grown in the presence of 20 mCi ^{32}P following the procedure of Garen and Levinthal [10]. The isolation of the ribosomes (to be published) and the synthesis of the affinity label were done as previously described [9]. 30S ribosomes were reactivated prior to use according to Kaufmann et al. [11].

2.2. *Ribosome labelling reaction*

The reaction mixture used for the labelling of the 30S ribosomes contained in a total volume of 350 μl: 200 pmol ^{32}P-labelled 30S ribosomes, 80 nmol oligonucleotide U-U-U-nhr$^{2'}$U, 300 pmol Phe-tRNA^{Phe}, 20 mM Mg $(OAc)_2$, 150 mM NH_4Cl and 50 mM Tris–Borate, pH 7.2. It was incubated at 0°C for 2 h.

2.3. *Separation of labelled 16S RNA*

After the incubation, the ribosomes were precipitated by the addition of 250 μl of ethanol (96%) at –20°C. The ribosomes were sedimented by centrifugation (Sorvall SE-12, 10 000 rev/min, 20 min) and dissolved in 200 μl buffer (150 mM NaCl, 15 mM Na-citrate, 10 mM EDTA-K, 50 mM Tris–Borate, pH 7.5, 1% SDS). They were extracted three times with

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200 μl phenol saturated with the same buffer. The water phases were combined and the RNA was precipitated by the addition of 2 volumes of 96% ethanol and kept at −20°C overnight. After centrifugation, SDS was removed by threefold extraction of the RNA pellet with ethanol (65%).

2.4. *Analysis of the labelled 16S RNA*

The analysis of the 16S RNA was carried out according to the diagonal fingerprint method described by Noller [12], because the reagent used has similar properties as kethoxal:

(1) It only reacts with guanine bases.
(2) Modified guanosines are resistant to T_1 RNase hydrolysis.
(3) The reagent can be removed by mild alkaline treatment (pH 9.8; 2 h; 37°C).

The first step of the procedure consists of a T_1 RNase hydrolysis in the presence of phosphatase giving rise to oligonucleotides with 3′-terminal guanosine (-G_{OH}). The products are separated by electrophoresis on DEAE-paper. During the second step the reagent is removed by alkaline treatment and the RNA is submitted to a second T_1 RNase hydrolysis but in the absence of phosphatase. On removal of the reagent, new guanosines become accessible to the RNase which are subsequently split to produce one oligonucleotide with 3′-Gp and one with 3′-G_{OH}. Every oligonucleotide with a 3′-Gp originates as a result of direct attachment of the reagent. The 3′-adjacent oligonucleotide ends with a 3′-G_{OH}.

Separation of these oligonucleotides in the second dimension was carried out under identical conditions as the first. The resulting new oligonucleotides appeared as spots which were located outside the diagonal line formed by all the unmodified oligonucleotides. Because the reagent contains four uridylic residues, the oligonucleotides with which it is associated remain on the origin during the first migration step, the resulting new spots are expected to be found on a line starting from the origin after the second migration step. The new spots were cut out and purified by electrophoresis on DEAE-paper at pH 1.9. The oligonucleotides were further analyzed by pancreatic and alkaline hydrolysis as described by Sanger et al. [13] and Brownlee and Sanger [14]. Digestion with ribonuclease U_2 was carried out as described by Ehresmann et al. [15].

3. Results

3.1. *Analysis of labelled oligonucleotides*

Five specific, reproducible oligonucleotides were obtained each time over a series of 10 experiments (fig.1). Table 1 shows the analysis of these oligonucleotides. Spot A was identified as spot 16 in the numbering system of Ehresmann et al. [16]. As confirmation, spot A showed an identical U_2-analysis pattern as spot 16 derived from the 16S RNA fingerprint and used as a marker.

3.2. *Localisation of the labelled oligonucleotides within the sequence of the 16S RNA*

Spot A which has the oligonucleotide sequence C-U-C-A-U-U-G_{OH}, occurs only once in the 16S RNA. This oligonucleotide is found in section I, about 450

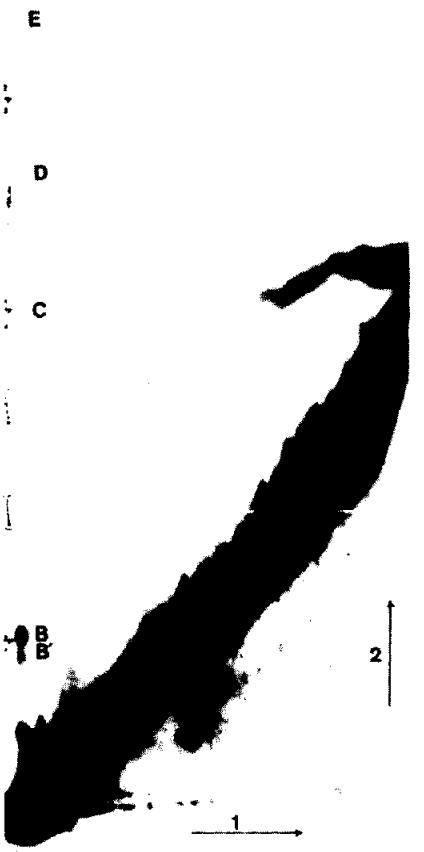

Fig.1. Autoradiography of a diagonal fingerprint of ^{32}P 16S RNA extracted from 30S subunits reacted with the messenger analogue in the presence of Phe-$tRNA^{Phe}$.

Table 1
Sequence analysis products from the oligonucleotides arising from the diagonal fingerprint as shown in fig.1.

Spot	Alkaline total hydrolysis products	Pancreatic RNase hydrolysis products	U_2 RNase hydrolysis products	Deduced sequence	No. according to Ehresmann et al. [16]
A	3Up, 2Cp, 1Ap	2Up, 2Cp, A-Up	U-U-G_{OH} (C_2U)Ap[a]	C-U-C-A-U-U-G_{OH}	16
B[b] B′	1Up, 3Ap, 1Gp	1Up, A-A-A-Gp	–	U-A-A-A-Gp	66
C	1Cp, 1Ap	Cp, A-G_{OH}	–	C-A-G_{OH}	97
D	1Ap, 1Gp	A-Gp	–	A-Gp	107
E	Gp	Gp	–	Gp	

[a] (C_2U) Ap runs exactly at the same position as C-U-C-Ap from spot 16.
[b] Sometimes oligonucleotide B gives rise to two spots B and B′ (as shown in fig.1) displaying different electrophoretic mobilities but identical nucleoside composition, terminating in 3′-guanylic acid and 2′, 3′-cyclic guanylic acid, respectively. The latter is probably due to incomplete T_1 ribonuclease digestion.

nucleotides away from the 5′-end of the 16S RNA. Spot B was identified as U-A-A-A-Gp (spot 66 according to Ehresmann et al. [16]). This sequence occurs twice in the 16S RNA, also being found in section I and in addition in section C″. As shown in fig.2, within section I the two oligonucleotides A and B are separated by only one T_1 RNase oligonucleotide, namely U-U-A-A-U-A-C-C-U-U-U-G (spot 1 according to Ehresmann et al. [16]). Because oligonucleotide 1 is adjacent to the 3′-end of oligonucleotide B and the 5′-end of oligonucleotide A, it should be found in either a 3′-phosphorylated or dephosphorylated form according the location of the modified G. However spot 1 was not observed on the diagonal fingerprint because it remains at the origin as a result of its high U residue content.

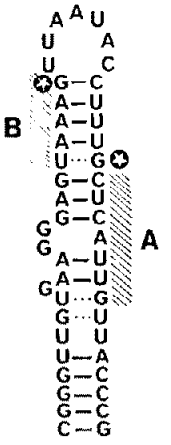

Fig.2. Localisation of the reactive guanosine within the section I as proposed by Ehresmann et al. [16]. Recent U_2 RNase analysis products from T_1 oligonucleotide 1 have been found to be in agreement with the sequence proposed by Magrum et al. [17] and the sequence has been corrected accordingly.

Since no other oligonucleotide in the 5′-direction from spot 66 was found from section C″, it is highly probable that spot B should be attributed to section I. Spots C, D and E do not represent characteristic oligonucleotides and for that reason they cannot be located unambiguously within the 16S RNA structure. Although they can all be located within section I, a region which can be clearly demonstrated as a direct binding site of the reagent, one cannot exclude the fact that spots C, D and E originate from other parts of the 16S RNA. This must be clarified by further analysis.

4. Discussion

Our results clearly demonstrate that section I of the 16S RNA either represents or is in the close proximity to the codon binding site of the 30S ribosome.

Section I represents part of the binding site for protein S_4 [18,19] and more recent information has demonstrated that this region may be directly linked to this protein by UV irradiation [20]. It is worth noting that protein S_4 may be involved in translational fidelity: protein S_4 has been shown to be altered in *E. coli* strains bearing the *ram* mutation [21] and in several revertants from streptomycin dependance [22]. These findings together with the fact that the A-U-G triplet analogue can be linked to protein S_4 [23] strongly suggest that protein S_4 may have a possible role in codon–anticodon recognition.

In addition, this region of the 16S RNA is well protected from chemical modification and enzymatic hydrolysis in the 30S ribosomes. Noller [12] could not localize guanine bases in section I that react with kethoxal. Since the mRNA analogue reacts specifically in the presence of tRNA with otherwise nonreactive guanosines, one has to entertain the possibility that a structural change in the I region occurs following the coded binding of a tRNA. However, it is not clear whether this accessibility is caused by a structural change in the 16S RNA or by an altered interaction between the I sequence and protein S_4. At the present we are investigating the protein neighbourhood of the I region with the use of the same affinity label.

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