AN ENZYMATIC APPROACH FOR LOCALIZATION OF OLIGODEOXYRIBONUCLEOTIDE BINDING SITES ON RNA

Application to studying rRNA topography

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1. Introduction

The method of oligonucleotide complementary binding has been widely used to study the macromolecular organization of RNAs [1]. In addition to probing the structure of RNA, it can also be used for site-specific affinity modification [2].

However, the complementary binding data are often hard or even impossible to interpret. Any high-molecular-mass (M_r) RNA has a number of binding sites which are partially complementary to any oligonucleotide used, whereas the region of total complementarity may well be hidden inside the RNA macromolecule.

Therefore, the fact that an oligonucleotide has hybridized with RNA is by no means unambiguous proof of the proper (i.e., total complementary) binding.

This paper describes a new approach to locating oligodeoxyribonucleotide binding sites on high-M_r RNA. It is based on the RNase H ability discovered by two of us (N. V. C. and A. A. B.) in collaboration with Metelev, Stepanova, and others [3–6] to cut RNA in the short oligodeoxyribonucleotide binding site, i.e., in the short DNA–RNA hybrid.

The data obtained by this method indicate unequivocally that the 5′-end region of 16 S rRNA is exposed on the 'surface' of both free rRNA and rRNA within the 30 S ribosomal subunit.

2. Materials and methods

The 30 S subunits and 16 S rRNA from *E. coli* MRE 600 were prepared as in [7]. RNase H (EC 3.1.4.34) free from other RNase activities was isolated from *E. coli* MRE 600 as in [8] and stored in a buffer containing 20 mM Tris–HCl (pH 7.9), 100 mM NaCl, 50 mM KCl, 0.1 mM DTT, 0.1 mM EDTA, 55% glycerol. All the oligonucleotides used (dCAAACT, dCAAACTCT, dCAAACTCTTCArA) were a generous gift of Drs S. Vinogradov and Yu. Berlin [9]. T4-polynucleotide kinase and ligase were products of SCTB BAP (Novosibirsk); *E. coli* alkaline phosphatase and RNase T_1 were purchased from Sigma, pancreatic RNase and RNase U_2 from Calbiochem, [α-^{32}P]ATP (>1000 Ci/mmol) from Amersham. [α-^{32}P]pCp (>1000 Ci/mmol) was synthesized from Cp as in [10].

2.1. *Hydrolysis of the free 16 S RNA*

16 S rRNA, 15 μg in 15 μl of 30 mM Tris–HCl (pH 8.0) were incubated with 0.03 μg alkaline phosphatase for 20 min at 37°C and then, after adding of EDTA up to 6.5 mM, heated for 3 min at 90°C. RNA was phosphorylated at the 5′-end with [α-^{32}P]ATP and T4-polynucleotide kinase as in [11]. The labeled RNA was deproteinized twice, precipitated with ethanol and dissolved in 8 μl of a buffer containing 10 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl, 5 mM DTT. Half of the RNA solution was treated with 2 μl of an oligonucleotide aqueous solution (25-fold molar excess) and 1 μl RNase H (act. 7 U/ml). The other half (control) was treated in the same way, but with 1 μl water instead of the oligonucleotide. After 1 h incubation at 20°C, the equal volume containing formamide, 0.05% xylene cyanol and bromophenol blue was added, the samples were heated for 1 min at 90°C and layered onto 20% polyacrylamide, 7 M urea, 50 mM Tris–borate (pH 8.3), 1 mM EDTA gel, 1 mm thick. After electrophoresis (40 V/cm) and

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radioautography, 16 S rRNA fragments were cut out and eluted from the gel as in [12]. RNA sequences of the 5′-end-labeled fragments were determined according to [11].

2.2. *Hydrolysis of the 16 S RNA in 30 S subunits*

30 S Ribosomal subunits, 9 μg in 5 μl of a buffer containing 20 mM Tris–HCl (pH 7.6), 20 mM $MgCl_2$, and 12 mM mercaptoethanol, were activated for 20 min at 37°C, then 2 μl of a dCAAACTCT aqueous solution (27-fold molar excess) and 5 μl RNase H (20–30 U/ml) were added, and the reaction lasted 14 h at 4°C. The reaction mixture was treated with phenol and precipitated with ethanol. 3′-Termini of 16 S rRNA fragments were labeled with [α-^{32}P]pCp and RNA ligase and the RNA fragments were subjected to electrophoresis in a 20% polyacrylamide gel as above. The RNA sequences of the 3′-end-labeled fragments were determined according to [12].

3. Results and discussion

Synthetic oligonucleotide dCAAACTCT, which is complementary to the 5′-end region of 16 S rRNA, binds with 16 S rRNA and with 30 S-ribosomal subunits and 70 S ribosomes with a relatively high and equal efficiency [13]. However, hybridization as such does not necessarily mean that the octamer binds with the 5′-end region of the 16 S rRNA.

To prove unequivocally that this is the case, the complex of the 30 S ribosomal subunit with dCAAACTCT, as well as the complexes of 16 S rRNA with oligonucleotides dCAAACT, dCAAACTCT, dCAAACTCTTCArA, which are complementary to the same 5′-end region of 16 S rRNA, were treated with RNase H. As seen in fig.1, this treatment yields rRNA fragments of different length, and this pattern of splitting is characteristic of each of the oligonucleotides used. The fragments were 5′-end-labeled, so it was rather convenient to sequence them by partial enzymatic cleavage. Fig.2 shows a sequencing gel for the RNA fragment produced by RNase H after the treatment of 16 S rRNA: dCAAACTCT complex. This fragment, as well as others (fig.1) appeared to be 5′-end fragments of 16 S rRNA. The longer the heteroduplex, the closer are the cutting points to the 5′-end of 16 S rRNA: hexa-, octa- and dodecamer give the fragments 12–13, 11–13 and 8–9 nucleotides long, respectively (fig.3). Another interesting observation is that each complex has several cutting points, the distribution of which does not depend on the duration of hydrolysis. The efficiency of cleavage in each point is the same between 5 - 60 min hydrolysis. The multiplicity of the cutting points within the complex was also pointed out in [14].

In the case of the 30 S ribosomal subunit, it is the complex with dCAAACTCT that was used for hydrolysis with RNase H. For this complex to be cleaved, the hydrolysis should be more vigorous (see section 2). As seen in fig.1,3, RNase H cuts this complex in the same fashion as the complex of the same oligonucleotide with free 16 S rRNA: in both cases RNase H gives a 16 S rRNA fragment 12 nucleotides long. Although the labeling of the reaction mixture was preformed after RNase H hydrolysis the radioactivity was found only in the small (5′-end proximal) fragments since

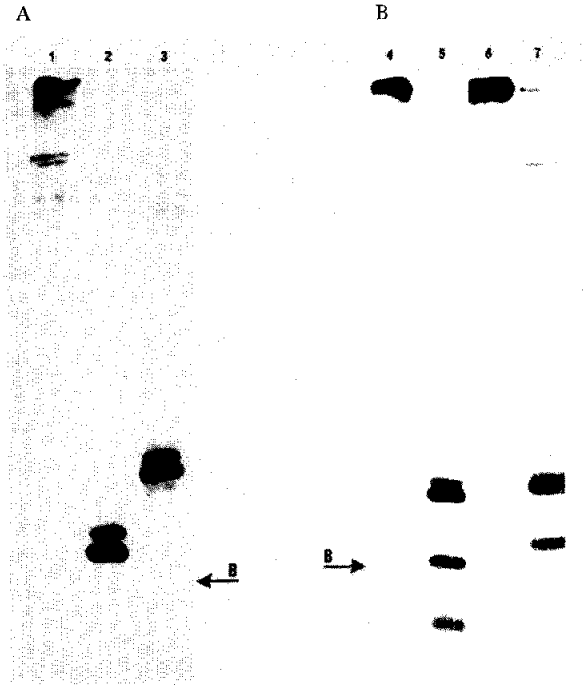

Fig. 1. A radioautograph of gel separation of RNase H cleavage products of the complexes of 5′-end-labeled 16 S rRNA with oligonucleotides (a, lanes 1–3) and of the 3′-end post-labeled rRNA fragments of the complexes of 30 S subunits and 16 S rRNA with dCAAACTCT (b, lanes 4–7). Lane: (1) (–) oligonucleotide, (+) RNase H; (2) (+) dCAAACTCTTCArA, (+) RNase H; (3) (+) dCAAACTCT, (+) RNase H, 20°C, 1 h; (4) (–) dCAAACTCT, (+) RNase H, (+) 16 S rRNA; (5) the same (+) oligonucleotide; (6) (–) oligonucleotide, (+) RNase H, (+) 30 S subunit; (7) the same (+) oligonucleotide, 4°C, 14 h. (B) denotes the position of bromophenol blue.

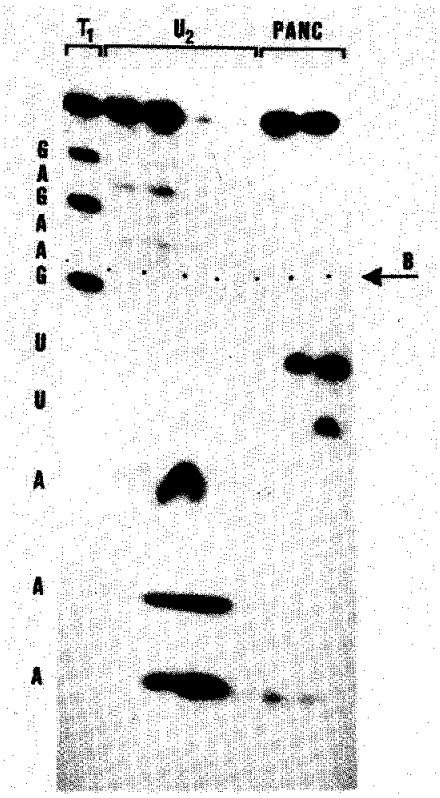

Fig.2. An example of the primary structure determination for 12-mer, a product of the cleavage of the complex of 5′-end-labeled 16 S rRNA with dCAAACTCT (fig.1). Base-specific partial cleavage of the fragment was made using RNase T_1 (G), U_2 (A) and pancreatic RNase (C + U). Pyrimidines were identified as U after the treatment with RNase Phy M. 20% polyacrylamide sequencing gel. (B) denotes the position of bromophenol blue.

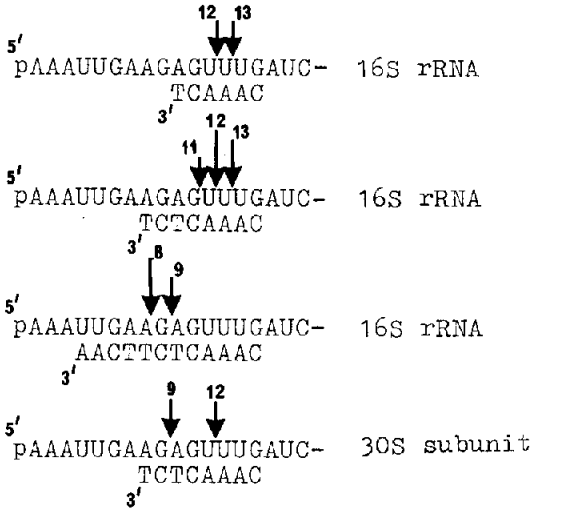

Fig.3. The diagram of the localization of cutting points for different complexes and different digestion conditions. The length of arrow corresponds to the yield of a rRNA fragment, the number above indicates the length of the rRNA fragment cleaved.

RNA-ligase shows a strong preference for the short substrates [10].

Two cleavage patterns differ in localization of additional cutting points. This may reflect the difference either in the conformation of the 5′-end of 16 S rRNA or in the digestion conditions. The latter assumption is confirmed by the hydrolysis of the complex of 16 S rRNA with dCAAACTCT under different conditions (fig.1, lanes 3,5).

Thus these data directly prove that the main binding sites of the synthetic oligodeoxyribonucleotides used are in the complementary sequences at the extreme 5′-end of 16 S rRNA, this region being exposed both in the compact free rRNA and in the 30 S ribosomal subunit.

A set of oligonucleotides of different length but complementary to one and the same RNA region is not yet sufficient for the mechanism of action of RNase H to be fully visualized. The mode of hydrolysis strongly depends on the digestion conditions: the conditions promoting stabilization of the complex allow RNase H to cut the RNA chain closer to the 3′-end of an oligodeoxyribonucleotide in a heteroduplex.

This approach can be generally used for direct locating the binding site(s) of oligodeoxyribonucleotide on high M_r RNA.

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