

The Ribosome-Binding Ability of Fragments of Bacteriophage f 2 RNA Carrying the 5'-terminus

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For experiment on the attachment site of ribosomes to bacteriophage f 2 RNA, the 5'-terminus of f 2 RNA was labeled with radioactive phosphate using polynucleotide kinase. The ribosome-binding and amino acid-incorporating activities of the labeled f 2 RNA were similar to those of intact f 2 RNA. The labeled f 2 RNA was treated with low concentrations of pancreatic RNase, and fragments of four different lengths carrying the original 5'-terminus were prepared by separating the radioactive fragments produced by sucrose density-gradient centrifugation. The approximate sizes of these fragments were 6s-7s, 10s-11s, 14s-15s and 20s-21s, respectively. When the ribosome-binding ability of these fragments was examined, the 14s-15s and 20s-21s fragments showed almost the same binding ability as the original strand. In contrast, the 6s-7s and 10s-11s fragments had little affinity for ribosomes. Possible interpretations of the above observation are discussed.

INTRODUCTION

There is good evidence for concluding that translation of the genetic message proceeds from the 5'- to 3'-end of messenger RNA; it is likely, in case of polycistronic messenger RNA such as bacteriophage f 2 RNA, that a ribosome first binds at the 5'-end of the RNA and moves along the strand, reading the message sequentially. However, this concept is complicated by the phenomenon that in infected *E. coli* cells, the phage coat protein does not appear before other proteins^{1,2}, whereas in a cell-free system the coat protein is synthesized predominantly and probably initially^{3,4}. An experiment was designed to find the initial attachment site of ribosomes or ribosomal subunits to the f 2 RNA-strand in order to explore the mechanism of regulation at the translational level. The primary approach was to label the 5'-end of the f 2 RNA molecule and to see whether the 5'-terminal region had or had not a special affinity for ribosomes. Since f 2 RNA was found to be phosphorylated at the 5'-terminus, it was treated with alkaline phosphatase and then re-phosphorylated with radioactive phosphate (³²P) by using polynucleotide kinase^{5,6}. It was found in the course of this study that the f 2 RNA molecule was broken into specific fragments by treatment with a very low concentration of pancreatic RNase, so that it became possible to prepare fragments of different lengths carrying the originally labeled 5'-terminus by treating the 5' (³²P)-f 2 RNA with RNase. In this communication, the ribosome-binding ability of such fragments will be described.

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MATERIALS AND METHODS

f 2 RNA: Bacteriophage f 2 was prepared by the method of Loeb and Zinder⁷⁾, except that *E. coli* A 19 was used as the host cell. RNA was extracted as described elsewhere⁹⁾. To obtain (³H)-f 2 RNA, the phage was grown in *E. coli* A 19 cultured in a Tris-glucose medium containing (³H)-uridine (5mC/liter) and caseamino acids (2 g/liter). RNA was stored in the presence of bentonite which was removed by centrifugation before use.

Ribosomes and their subunits: Ribosomes were extracted from *E. coli* B, grown in a M 9-medium, using 10 mM Mg⁺⁺ buffer (10 mM Mg-acetate, 50 mM NH₄Cl, 5 mM mercaptoethanol, 20 mM Tris-HCl, pH 7.6) and were washed by centrifugation at 40,000 rev/min for 2 h. The ribosomal pellet obtained was dissolved in 0.25mM Mg⁺⁺ buffer (0.25 mM Mg-acetate, 50 mM NH₄Cl, 5 mM mercaptoethanol, 20 mM Tris-HCl, pH 7.6) and then passed through a Sephadex G 200 column (70 cm long) equilibrated with the same buffer. The dissociated ribosomal fraction eluted was dialyzed against 10 mM Mg⁺⁺ buffer to give the "recombined ribosomes". Sedimentation analysis of the dialyzed fraction showed to contain mostly the 70 s particle. To prepare the ribosomal subunits, the dissociated ribosomal fraction was placed on a 5-20% sucrose density-gradient prepared in 0.25 mM Mg⁺⁺ buffer and centrifuged for 8 h at 25,000 rev/min. The respective 30 s and 50 s fractions were collected and dialyzed against 10 mM Mg⁺⁺ buffer. The molar amount of ribosomes was calculated assuming a particle weight of 2.6×10⁶, 1.8×10⁶ and 0.7×10⁶ daltons per 70 s, 50 s and 30 s particles, respectively, and an extinction coefficient of 18A₂₆₀ units/mg.

(γ ³²P)-ATP: 10⁹ to 10¹⁰ counts/min/ μ mole of (γ ³²P)-ATP was prepared as described by Glynn and Chappell¹⁰⁾.

Enzymes: Polynucleotide kinase and alkaline phosphatase were purified free of contaminating endonuclease by procedures described previously⁵⁾.

Preparation of f 2 RNA containing ³²P at the 5'-hydroxyl end: About 20 mg of f 2 RNA was incubated at 37°C with 1,000 units⁵⁾ of alkaline phosphatase in the presence of 5 mM Mg⁺⁺ and 0.1 M Tris-HCl, pH 7.6. After 2 h, 600 units of phosphatase were added and the incubation was continued for another hour. The reaction mixture was treated with phenol to remove the enzyme and then layered on a sucrose density-gradient (5-20% in 0.1 M NaCl-0.02 M Tris-HCl, pH 7.6). After centrifugation for 15 h at 25,000 rev/min and 5°C, the peak region of A₂₆₀ was collected. For rephosphorylation of the 5'-terminus with ³²P, 2 mg of phosphatase-treated RNA was incubated for 3 h with 100 units⁵⁾ of polynucleotide kinase in the presence of 200 m μ moles (γ ³²P)-ATP, 10 mM Mg⁺⁺, 10 mM mercaptoethanol and 50 mM Tris-HCl, pH 7.6. The phosphorylated product (designated as 5' (³²P)-f 2 RNA) was isolated from the reaction mixture as previously described⁵⁾. During phosphorylation, the amount of ³²P transferred to the 5'-terminus reached about 0.8 m μ moles/mg RNA, corresponding to about 90% of the terminus as calculated from a molecular weight of 1.1×10⁶. The preparation thus obtained was mixed with intact f 2 RNA uniformly labeled with ³H, and the mixture was analysed by sucrose density-gradient centrifugation. As shown in Fig. 1, the radioactivity

peaks of ^{32}P and ^3H appeared in the same region.

Assay of the ribosome-binding ability: This was by a millipore filter technique, described by Moore¹¹⁾ and Dahlberg and Haselkorn¹²⁾. Unless otherwise indicated, the reaction mixture contained in a final volume of 0.5 to 1.0 ml, 10 mM Mg^{++} , 50 mM NH_4Cl , 50 mM Tris-HCl, pH 7.6, 20 $\mu\mu\text{moles}$ ribosomes or ribosomal subunits and varying amounts of radioactive RNA. The mixture was incubated for 10 min at 25°C, then diluted with about 5 volumes of cold 10 mM Mg^{++} buffer, and poured onto a millipore filter (HA 0.45 μ , 25 mm in diameter). The filter was washed three times with 5 ml portions of 10 mM Mg^{++} buffer, dried and the radioactivity was counted. Neither f2 RNA nor fragments of f2 RNA adsorbed on the filter, whereas about 7 A_{260} units of ribosomes or ribosomal subunits could be adsorbed on the filter at Mg^{++} concentrations ranging from 0.25 mM to 30 mM. The effect of the Mg^{++} concentration on the binding of f2 RNA to ribosomes was examined and it was observed that the maximum binding occurred at 5-10 mM Mg^{++} (Fig. 2). Efficiency of binding of f2 RNA to ribosomes was not high under the conditions described above (see Fig. 4), though it was affected by the mixing conditions and also varied with preparations of ribosomes. This implies that a small fraction of ribosomes in preparations was active for the binding.

In vitro protein synthesis: Protein synthesis in cell extracts was carried out by the procedure described by Capecchi¹³⁾.

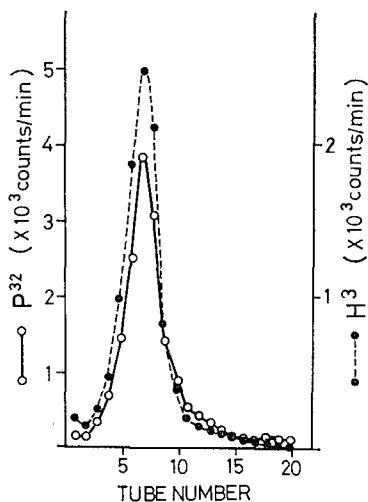


Fig. 1. Sucrose density-gradient centrifugation analysis of f2 RNA phosphorylated with ^{32}P at the 5'-terminus.

0.5 mg of f2 RNA phosphorylated with ^{32}P (3.5×10^5 counts/min/mg) was mixed with 0.1 mg of intact f2 RNA uniformly labeled with ^3H and the mixture was spun for 16 h at 25,000 rev./min and 5°C.

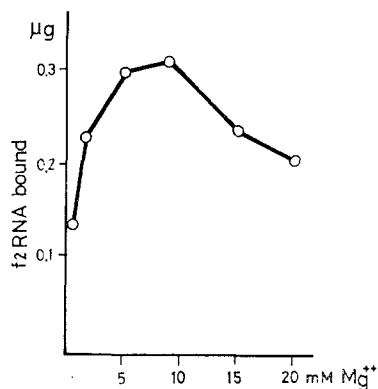


Fig. 2. The effect of Mg^{++} concentration on the binding of f2 RNA to ribosomes.

20 $\mu\mu\text{moles}$ of ribosomes were mixed with 20 μg of ^3H -f2 RNA in 50 mM NH_4Cl -50 mM Tris-HCl, pH 7.6 and the Mg -concentration was adjusted as indicated. After incubation for 10 min at 25°C, the amount bound to ribosomes was assayed by means of the milliporefilter technique.

Ribosome-Binding by f2 RNA Fragments

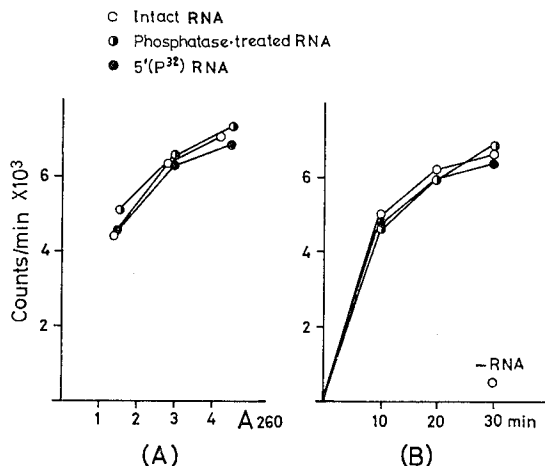


Fig. 3. The amino acid-incorporating activity of intact f2 RNA compared with preparations from which the terminal phosphate had been removed and re-phosphorylated using polynucleotide kinase.

The template activity of the phosphatase-treated f2 RNA and that re-phosphorylated using polynucleotide kinase was compared with that of intact f2 RNA in a cell-free amino acid-incorporating system described by Capecchi¹⁴. ¹⁴C-valine (10⁴ counts/min/mμmole) was used as the radioactive amino acid. In (A), increasing amounts of RNA were added to the reaction mixtures. Following incubation for 30 min, the radioactivity incorporated was determined. In (B), 3A₂₆₀ units of RNA were added to each tube and kinetics of the incorporation was studied.

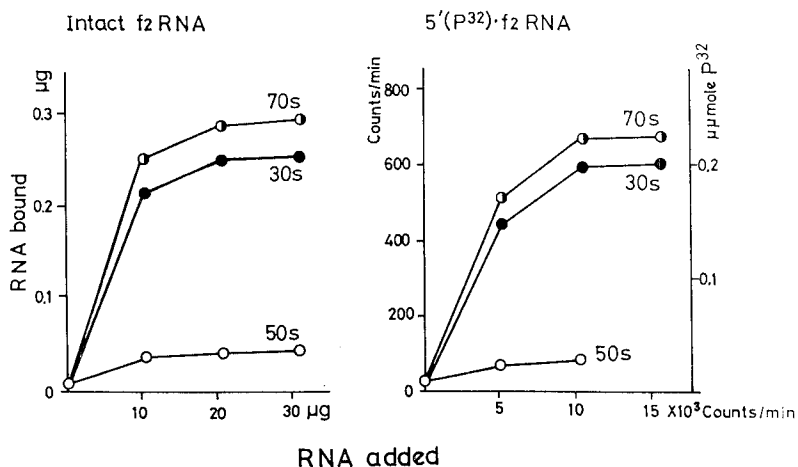


Fig. 4. Comparison of the ribosome-binding ability of intact f2 RNA and 5' (³²P):f2 RNA.

The intact f2 RNA uniformly labeled with ³H (6.2×10⁵ counts/min/mg) or 5' (³²P)-f2 RNA (2.7×10⁵ counts/min/mg) was mixed with either 20 μμmoles of the 70 s particle or ribosomal subunits, and the amount bound to ribosomes was assayed as described in the text. The 70 s particle used for experiment was prepared as follows: the recombined ribosomal fraction was placed on a sucrose densitygradient (5%-20% in 10 mM Mg⁺⁺ buffer) and centrifuged for 6 h at 25,000 rev/min to isolate the 70 s region.

RESULTS

Amino acid-incorporating and ribosome-binding abilities of 5' (³²P)-f2 RNA :

Since the intact f2 RNA molecule appeared to be terminated by a nucleoside triphosphate at the 5'-end⁶⁾, the untreated and 5' (³²P)-RNA differ with respect to the number of phosphates at the 5'-terminus. The template activity of these RNAs was compared using a cell-free amino acid-incorporating system prepared from *E. coli*. As shown in *Fig. 3*, the phosphatase treated f2 RNA and the 5' (³²P)-f2 RNA showed amino acid-incorporating activity similar to that of the intact f2 RNA. The ability of the 5' (³²P)-f2 RNA and the untreated RNA to bind ribosomes were compared using the millipore filter technique and no significant difference was found. As shown in *Fig. 4*, the binding profiles of the two RNA's to the 30s subunit was almost identical. Reformation of the 70s particle by adding the 50s subunit did not affect the messenger RNA-binding ability, showing that messenger RNA binds to the 30s subunit independently of the attachment of the 50s subunit^{9,14)}.

Preparation of f2 RNA-fragments carrying the 5'-terminus : The high molecular weight RNA is typically very fragile and readily degraded into fragments by treating it with a very low concentration of pancreatic RNase. The degradation of f2 RNA apparently was not random, however. Two fragments with sedimentation rates of roughly 20s and 14s always appeared as the first step in the degradation. Degradation proceeded further when the treatment with RNase was prolonged and finally a broad peak of 5s-8s was produced. A similar phenomenon was described by Bassel and Spiegelman¹⁵⁾ with phage Q β RNA and by Fiers¹⁶⁾ with phage MS 2 RNA.

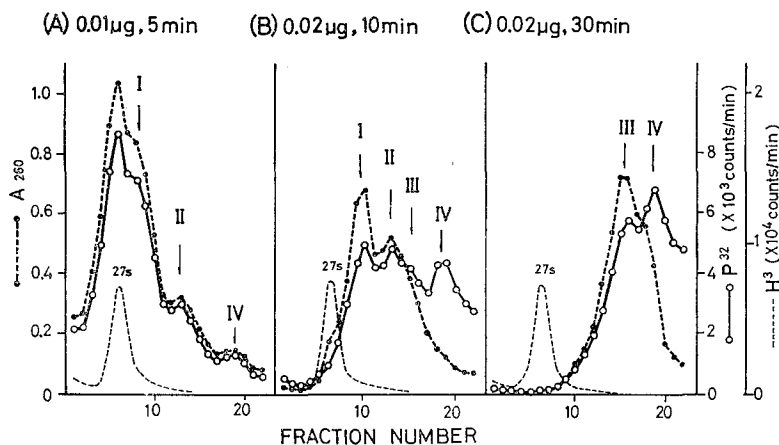


Fig. 5. Degradation of 5' (³²P)-f2 RNA by pancreatic RNase.

0.5 mg of 5' (³²P)-f2 RNA (2.5×10^5 counts/min/mg) was dissolved in 0.005 M Mg⁺⁺-0.1 M NaCl-0.02 M Tris-HCl, pH 7.6 for each experiment, and treated at 37°C with pancreatic RNase as indicated. The reaction was terminated by adding 2 mg bentonite. Intact f2 RNA labeled with ³H was added as marker, and the mixture was spun at 25,000 rev./min for 16 h on a sucrose density-gradient (5-20% in 0.1 M NaCl-0.02 M Tris-HCl, pH 7.6).

Ribosome-Binding by f 2 RNA Fragments

Applying the principle described above, different lengths of fragments carrying the 5'-terminus were prepared by treating the 5' (^{32}P)-f 2 RNA with low concentrations of pancreatic RNase. Results of a typical experiment are shown in Fig. 5. Under very mild conditions, three radioactive peaks appeared in addition to the original (A in Fig. 5). Almost all the radioactivity originally located at the 27 s region shifted to lighter regions, when the time was prolonged and the enzyme concentration was doubled (B and C in Fig. 5). The respective regions indicated by arrows in the figures (F-I, F-II, F-III and F-IV) were pooled, concentrated and recentrifuged. The sedimentation profiles of the respective fractions obtained by the second centrifugation are shown in Fig. 6. The S value at the peak position was estimated to be roughly 20 s-21 s, 14 s-15 s, 10 s-11 s and 6 s-7 s for F-I, F-II, F-III and F-IV, respectively, comparing their sedimentation rates with those of *E. coli* ribosomal RNA as marker. The approximate size was estimated by using an empirical equation: $S \propto M^{0.49}$ (S =sedimentation coefficient, M =molecular weight). Assuming that the original 27 s molecule had a molecular weight of 1.1×10^6 , F-I would correspond to roughly 2/3, F-II to 1/3, F-III to 1/6, and F-IV to 1/15 of the chain starting from the 5'-end of the f 2 RNA strand.

The ribosome-binding ability of the ^{32}P -terminated fragments: The ribosome-binding ability of different lengths of fragments prepared as above was examined by means of the millipore filter technique. Almost identical results were obtained with recombined ribosomes and the 30 s subunit. As shown in Fig. 7, the F-I

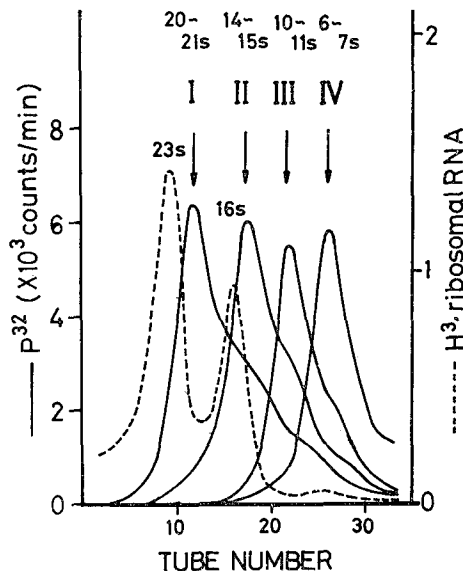


Fig. 6. Sucrose density-gradient centrifugation analysis of the fractions containing different lengths of radioactive fragments.

The fractions indicated by arrows in Fig. 5 (I, II, III and IV) were pooled, concentrated and recentrifuged on sucrose density gradients as in Fig. 5. The ^{32}P -peak regions obtained by the second centrifugation was collected. Aliquots were analysed by sucrose density-gradient centrifugation (25,000 rev./min, 18 h) together with marker (^3H)-ribosomal RNA from *E. coli*. The relative ^{32}P -profiles of the respective fractions to ribosomal RNA were plotted for comparison. The approximate S values of the peak positions are indicated in the figure.

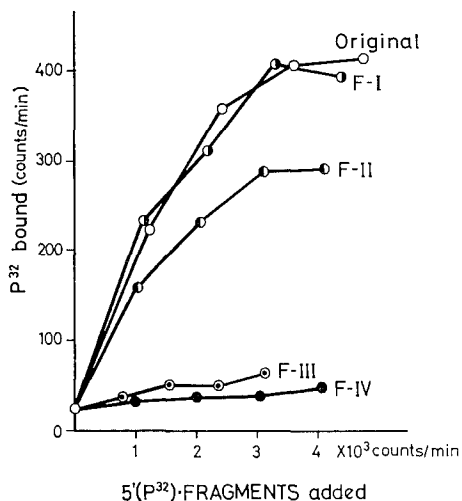


Fig. 7. The ribosome-binding ability of different lengths of the f2 RNA-fragments carrying the 5'-terminal ³²P.

The reaction mixture contained in a final volume of 1 ml, 20 μ moles of recombined ribosomes, 10 mM Mg⁺⁺, 50 mM NH₄Cl, 50 mM Tris-HCl, pH 7.6, and increasing amounts of fragments carrying ³²P at the 5'-terminus (Fraction I, II, III and IV in Fig. 6). The radioactivity bound to ribosomes was assayed following incubation for 10 min at 25°C.

and F-II fragments which contained about 2/3 and 1/3 of the chain from the 5'-end had almost the same binding ability as the original strand. In contrast, the F-III and F-IV fractions showed little affinity for ribosomes.

DISCUSSION

The f2 RNA molecule is assumed to contain at least three cistrons, including those for coat-protein, RNA-synthetase and "maturation" protein⁴). When f2 RNA was added to a cell-free protein synthesizing system, however, the coat protein was synthesized predominantly and initially^{3,4}). It has been observed that the mixture of f2 RNA and ribosomes does not produce heavier complexes, but forms a complex corresponding to the size of "monosome"^{8,18}). Therefore, it is likely that a ribosome first binds to the f2 RNA-strand at the starting point of the cistron for coat-protein, though little is known of how the ribosome recognizes such the specific region. In the present study, fragments of four different lengths carrying the original 5'-terminus of f2 RNA were prepared and their ribosome-binding ability was compared with that of the original strand. As the result, it was found that the F-III and F-IV fragments, which contained about 1/6 and 1/15 of the chain from the 5'-terminus respectively, did not bind ribosomes, in contrast to the other longer fragments. Assuming that one ribosome can bind per f2 RNA-strand, the result appears to indicate that the *in vitro* ribosome-binding site was not at or near the 5'-terminal region, but at an internal region of the f2 RNA-strand. Since the F-II fragment which contained about 1/3 of the chain from the 5'-end showed almost the same binding ability as the original strand, such the binding site may be about 15% to 30% of the distance along the f2 RNA molecule starting from the 5'-terminus.

Ribosome-Binding by f2 RNA Fragments

An alternative interpretation should be considered. According to my previous observations¹⁴⁾ and Szer and Nowak¹⁷⁾, polynucleotides become attached to ribosomes above a critical temperature consistent with its transition from an ordered state to a random coil. The binding of f2 RNA to ribosomes was compatible with binding of poly-U, in agreement with observations on turnip yellow mosaic virus RNA¹²⁾. These observations led a speculation that the specific ribosome-binding site is formed only by the three-dimensional configuration of the f2 RNA molecule in solution and that disruption of the configuration by breaking the strand may destroy its ability to bind ribosomes. In this case, even if it contains the initial sequence of a cistron, the fragment might not show particular affinity to ribosomes.

As has been also observed with Q β RNA¹⁵⁾, fragments of specific length were produced by exposing f2 RNA to very low concentration of pancreatic RNase. Since f2 RNA in solution shows a high T_m value in the presence of Mg⁺⁺ upon heating⁸⁾, it is likely that the f2 RNA molecule in solution forms a secondary structure that is quite compact but contains specific regions having configuration which are vulnerable to endonuclease.

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ABBREVIATIONS

RNA: ribonucleic acid, ATP: adenosine triphosphate, 5' (³²P)-RNA: RNA containing radioactive phosphomonoester at the 5'-hydroxyl end.

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