*ON THE NATURE OF TWO RIBOSOMAL SITES FOR SPECIFIC SRNA BINDING**

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It is now well established that 30*S* ribosomal subunits alone can bind specific sRNA or aminoacyl sRNA in the presence of the corresponding messenger RNA.[1-4] Although 50*S* subunits alone cannot bind specific sRNA they stimulate the binding of specific sRNA about twofold when associated with 30*S* ribosomal subunits.[5, 6] In this communication we present evidence supporting the notion that two sRNA molecules are bound to a 70*S* ribosome. These two sites are named site 1 (COOH side of the growing polypeptide chain) and site 2 (NH_2 side of the growing polypeptide chain). Site 1 has about three times more affinity for sRNA than has site 2.

Materials and Methods.—E. coli extract and other materials: Preparation of ribosomes, sRNA from *E. coli* B, and aminoacyl sRNA have been described in the preceding communications.[1, 2, 5, 7] In some cases strain Q13 of *E. coli* was used. The ribosomes were washed 3 times and were free of the aminoacyl sRNA transfer factor. For preparation of *E. coli* soluble protein fraction, the Is-30 of Nirenberg and Matthaei[8] was centrifuged for 90 min at 150,000 *g* and the supernatant fluid was dialyzed overnight against a buffer containing 0.01 *M* Tris-HCl (pH 7.8), 0.01 *M* magnesium acetate, 0.006 *M* β-mercaptoethanol, and 0.06 *M* KCl (Buffer 1). This fraction was called S-150. Specific radioactivities of materials used in this paper were as follows: C^{14}-phenylalanine, 395 μc/μmole; H^3-phenylalanine, 2800 μc/μmole. Counting efficiency was $1.0 \sim 1.5 \times 10^6$ cpm/μc and $1.0 \sim 1.8 \times 10^5$ cpm/μc for C^{14} and H^3, respectively. When C^{14} and H^3 were counted simultaneously, the counting efficiency for C^{14} was $1.8 \sim 6.2 \times 10^5$ cpm/μc.

Reaction mixture for the binding of C^{14}-phenylalanyl sRNA: A typical reaction mixture for phenylalanyl sRNA binding contained the following in μmoles per 2.05 ml: 73.8 Tris-HCl (pH 7.1), 30.0 magnesium acetate, and 39.4 KCl. In addition, it contained 400 μg of poly U (ammonium salt) and C^{14}-phenylalanyl sRNA and 70*S* ribosomes. Incubation was carried out for 20 min at 22°C. In some cases bound C^{14}-phenylalanyl-sRNA was measured by the method of Nirenberg and Leder.[9]

Isolation of the ribosome C^{14}-phenylalanyl-sRNA-poly U complex and formation of polyphenylalanine: After the binding of C^{14}-phenylalanyl sRNA to 70*S* ribosomes was completed, the reaction mixture was centrifuged for 1.5 hr at 150,000 *g*. The pellet was suspended in 0.2 ml of Buffer 1. For polyphenylalanine synthesis the reaction mixture contained the following in μmoles in a total volume of 1.20 ml: 56.2 Tris-HCl (pH 7.8), 23.0 magnesium acetate, 53.8 KCl, 4.8 β-mercaptoethanol, 1.3 phosphoenolpyruvate (Na-salt), and 0.07 GTP. In addition, it contained 3 mg of S-150 protein, 34 μg of pyruvate kinase, 20 mg of a mixture of sRNA in which phenylalanine specific sRNA is aminoacylated with C^{12}-phenylalanine, and 0.2 ml of the resuspended pellet described above. The mixture was incubated at 37°C for 15 min.

NH_2-terminal analysis of polyphenylalanine: At the end of the incubation period, an *E. coli* soluble protein fraction (Fraction A of ref. (7) was added to the reaction mixture to make the total protein content 5 mg. Immediately after the addition of the soluble protein, 0.2 ml of 0.1 *M* phenylalanine solution was added followed by 10% trichloracetic acid to a final concentration of 5%. The precipitate containing polyphenylalanyl sRNA was treated with hot trichloracetic acid, a mixture of ether-alcohol and with ether as described previously.[10] The precipitate was mixed with 1 ml of 5% $NaHCO_3$ and 2.0 ml of 5% dinitrofluorobenzene (DNFB) in alcohol. The mixture was shaken at room temperature overnight and 50% trichloroacetic acid was added to a final concentration of 5%. The precipitate was collected and washed with ether to remove excess DNFB. The yellow precipitate was mixed with 1.5 ml of 6 *N* HCl and hydrolysis was carried out at 115–118° for 20 hr under a N_2 atmosphere. After hydrolysis, the mixture was diluted with 3 ml of water and dinitrophenyl (DNP) phenylalanine was extracted with three 5 ml of ether. The ether

phase and the aqueous phase were counted for radioactivity. It is important to compensate for the quenching effect of DNP-amino acids, protein, etc. on the scintillation counting of the samples. Radioactivity in the ether layer was identified as DNP-C^{14}-phenylalanine by paper chromatography (Fig. 1*A*). Less than 2.0% of C^{14}-DNP-phenylalanine was decomposed during the hydrolysis of DNP-polyphenylalanine. As shown in Figure 1*B*, the radioactivity in the aqueous layer was identified as C^{14}-phenylalanine.

Results.—Preferential incorporation of bound C^{14}-phenylalanyl sRNA into polyphenylalanine: Despite numerous studies carried out on the specific binding of aminoacyl sRNA to ribosomes, the exact relationship between the binding process and peptide bond formation has remained obscure. In the experiment shown in Figure 2, complex of C^{14}-phenylalanyl sRNA, ribosomes and poly U was mixed with H^3-phenylalanyl sRNA and the other components of the polyphenylalanine synthesis system. As shown in this figure, the ratio of H^3 to C^{14} in the polyphenylalanine increased as the time of incubation proceeded. The data show that the C^{14}-phenylalanine from bound C^{14}-phenylalanyl sRNA is preferentially incorporated at the initial stage of polyphenylalanine synthesis. This indicates that the bound C^{14}-phenylalanyl sRNA participates first in the polypeptide synthesis followed by the chain elongation with H^3-phenylalanine.

Evidence for two sRNA binding sites and their relative affinity for phenylalanyl sRNA: Although evidence has been accumulating that there are two ribosomal sites for specific binding of phenylalanyl sRNA[11–13] definite chemical evidence has not been obtained to prove this point. If two molecules of C^{14}-phenylalanyl

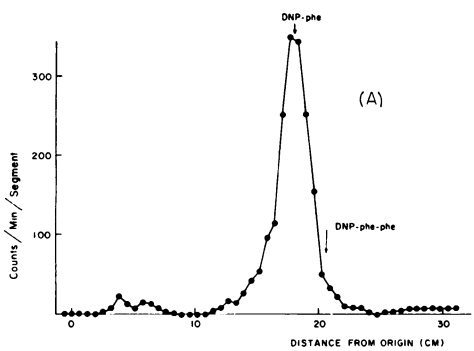

FIG. 1*A*.—Identification of radioactivity in ether phase by paper chromatography. Uniformly labeled C^{14}-polyphenylalanine (120,000 cpm) was processed for NH_2-terminal analysis as described in the text. The ether extract (15 ml) containing DNP-phenylalanine was evaporated, and the residue was dissolved in 0.3 ml of acetone. The solution (0.05 ml containing 2200 cpm) was streaked (2.5 cm length) on Whatman no. 1 filter paper. Ascending paper chromatography was carried out for 13 hr at room temperature with a solvent containing toluene pyridine, and 2-chloroethanol (10:3:6 v/v), which had been equilibrated with 0.8 *N* NH_4OH for 4 hr at room temperature. The paper was dried at 40°C for 1 hr. A 4-cm-wide vertical strip was cut along the direction of solvent flow, and this strip was further cut into horizontal segments of 0.64 cm each. Each paper segment was counted.

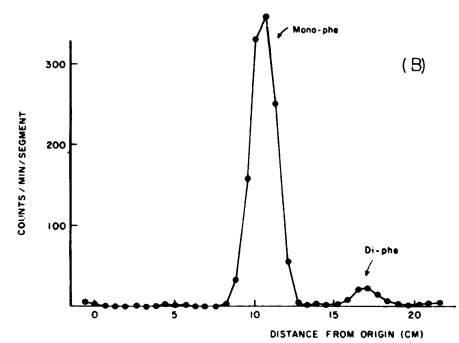

FIG. 1*B*.—Identification of radioactivity in aqueous phase by paper chromatography. The aqueous phase (4.5 ml) prepared as in (*A*) was lyophilized, and the residue was dissolved in 4.5 ml of water and lyophilized. The procedure was repeated 4 times to remove HCl. The final residue was dissolved in water and 0.05 ml of this solution containing 1600 cpm of radioactivity was streaked on Whatman no. 1 filter paper. Ascending paper chromatography was carried out for 13 hr at room temperature with a solvent containing 1-butanol, acetic acid, and water (90:10:25). About 95% of radioactivity was recovered at the position where standard phenylalanine was located.

sRNA are bound to a ribosome and the polyphenylalanine formation is started from this bound sRNA as indicated in the preceding section, one would expect that one of the two phenylalanines which were bound initially would be located at the NH_2-terminal end of the polyphenylalanine. In the experiment shown in Figure 3, the complex of C^{14}-phenylalanyl sRNA, poly U, and ribosomes was isolated and polyphenylalanine was made from this complex by the addition of excess C^{12}-phenylalanyl sRNA and other components for polypeptide synthesis. Since the polypeptide chain is elongated from the NH_2-terminal to the COOH-terminal, the first and the second phenylalanine from the NH_2-terminal should be labeled with C^{14} and the rest of the polyphenylalanine should contain C^{12}-phenylalanine. Therefore, 50 per cent of the total radioactivity should be found at the NH_2-terminal. This was indeed the case when excess C^{14}-phenylalanyl sRNA was present in the mixture for sRNA binding. The ratio of radioactivity found in the non-NH_2-terminal amino acid to that found in the NH_2-terminal was approximately 1. This finding strongly supports the concept that two sRNA molecules are bound per one ribosome. It is noted in this figure that the ratio of radioactivity between non-NH_2-terminal and NH_2-terminal position starts increasing at the point where molar ratio of ribo-

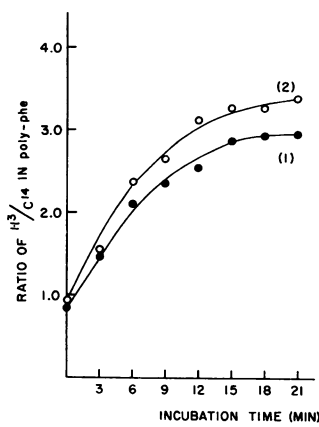

FIG. 2.—Initial preferential incorporation of C^{14}-phenylalanine from ribosome-bound C^{14}-phenylalanyl sRNA. The reaction mixture (2.05 ml) for the binding of C^{14}-phenylalanyl sRNA contained, in addition to the components described in the text, 220,000 cpm of C^{14}-phenylalanyl sRNA and 900 μg of 70*S* ribosomes. After the binding was completed, the complex was isolated and suspended in 0.2 ml of Buffer 1. The reaction mixture (0.60 ml) contained, in addition to the components listed in the text, 0.1 ml (1), or 0.03 ml (2) of the suspension of the complex, and 155,000 cpm of H^3-phenylalanyl sRNA and 2480 $\mu\mu$moles of C^{12}-phenylalanyl sRNA. At the time intervals indicated, aliquots (0.06 ml) were taken, and C^{14} and H^3 radioactivity in polyphenylalanine were measured. At 21 min after the onset of incubation 3418 cpm (1), and 1021 cpm (2) of H^3-phenylalanine were incorporated into polyphenylalanine per 0.06 ml of the reaction mixture.

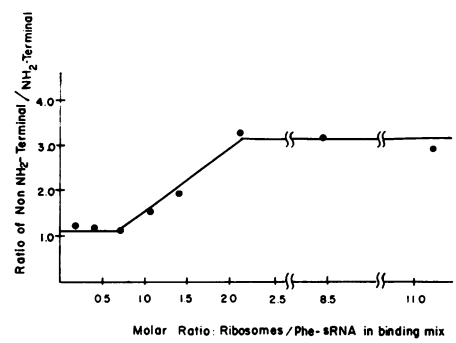

FIG. 3.—Position in polyphenylalanine of C^{14}-phenylalanine derived from initially bound C^{14}-phenylalanyl sRNA. The reaction mixture (2.05 ml) contained, in addition to the components listed in the text, various amounts of C^{14}-phenylalanyl sRNA and 70*S* ribosomes to obtain the molar ratios of C^{14}-Phe-sRNA to ribosomes as described in the text in a total volume of 1.2 ml. After the NH_2-terminal analysis of the polyphenylalanine, the ratio of radioactivity between non-NH_2-terminal/NH_2-terminal was determined for each sample and plotted against the molar ratio of ribosomes to C^{14}-phenylalanyl sRNA in the binding mixture.

somes to C^{14}-phenylalanyl sRNA is about 0.8 instead of 0.5. This is due to the fact that the ribosome preparation contains some inactive ribosomes and not all the phenylalanyl sRNA in the reaction mixture for binding is bound to ribosomes under these conditions.

Of the two sRNA binding sites one site may have easier access to sRNA than the other. This possibility was tested by varying the concentration of ribosomes in the reaction mixture for binding of C^{14}-phenylalanyl sRNA. In the presence of excess ribosomes, there was not enough phenylalanyl sRNA in the reaction mixture to fill all the sites available. Consequently the site which has easier access to sRNA would more likely be occupied by C^{14}-phenylalanyl sRNA. In the experiment shown in Figure 3, the relative radioactivity found at the NH_2-terminal of the polyphenylalanine decreased as the amount of ribosomes in the binding mixture increased. Thus, the ratio of radioactivity of non-NH_2-terminal phenylalanine to that of NH_2-terminal phenylalanine gradually increased until it reached the value of three. At this point, further increase of ribosome concentration did not influence the ratio. These results suggest that relative affinity of site 1 to site 2 for phenylalanyl sRNA is approximately three.

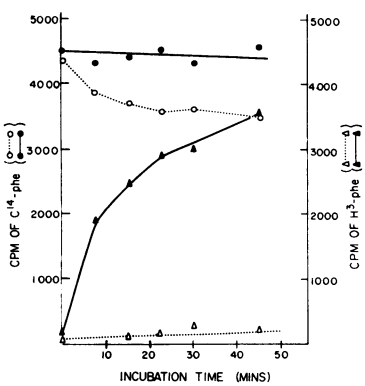

FIG. 4.—Absence of release of originally bound C^{14}-phenylalanyl sRNA during polyphenylalanine synthesis. The reaction mixture (2.05 ml) for the binding of C^{14}-phenylalanyl sRNA contained, in addition to the basic components listed in the text, 715,000 cpm of C^{14}-phenylalanyl sRNA and 1.6 mg of 70*S* ribosomes. The complex of C^{14}-phenylalanyl sRNA, ribosomes and poly U was isolated and polyphenylalanine was synthesized from this complex in a total volume of 0.60 ml as described in the text except that 187,500 cpm of H^3-phenylalanyl sRNA and 2110 $\mu\mu$mole of C^{12}-phenylalanyl sRNA and 0.8 mg of the complex of C^{14}-phenylalanyl sRNA, ribosomes and poly U were used. At the intervals 0.06 ml of the reaction mixture was taken and mixed with 1.0 ml of buffer containing 0.1 *M* Tris-HCl (pH 7.1), 0.02 *M* magnesium acetate and 0.05 *M* KCl. The mixture was poured onto a prewashed Millipore filter and the filter was washed with three 1-ml fractions of the same buffer and the filter paper was counted. Ribosomes are retained by the filter.[9] ●—●, C^{14} radioactivity bound on the ribosomes; (complete reaction mixture for polyphenylalanine formation). O---O, C^{14} radioactivity bound on the ribosomes; (soluble enzymes and GTP were omitted from the reaction mixture for polyphenylalanine formation). ▲—▲, H^3 radioactivity on the ribosome (complete system). △---△, H^3 radioactivity on the ribosomes; (soluble enzymes and GTP were omitted from the reaction mixture for polyphenylalanine formation).

Absence of release of bound phenylalanyl sRNA: The experiment described in the preceding section was constructed in such a way that the bound C^{14}-phenylalanyl sRNA could not be reincorporated into polyphenylalanine if it were accidentally released from ribosomes. Thus, if C^{14}-phenylalanyl sRNA is released from ribosomes before it is incorporated into polypeptide, the released C^{14}-phenylalanyl sRNA is diluted by the presence of large excess of C^{12}-phenylalanyl sRNA and practically no possibility exists that this C^{14}-phenylalanine is later incorporated into polyphenylalanine. On the other hand, it was of interest to examine how much of C^{14}-phenylalanine of initially bound phenylalanyl sRNA is released from ribosomes during polypeptide synthesis. Figure 4 shows that no release takes

place during the polypeptide synthesis. In this experiment, a complex of C^{14}-phenylalanyl sRNA, ribosomes and poly U were mixed with a large excess of H^3-phenylalanyl sRNA and polyphenylalanine synthesis was allowed to proceed. As shown in this figure in the presence of soluble enzymes and GTP, very little, if any reduction of C^{14}-radioactivity on the ribosome took place, whereas a steady increase of H^3-radioactivity on the ribosome was observed. On the other hand, a small amount of loss of C^{14}-radioactivity was observed in the absence of polypeptide synthesis while no increase of H^3-radioactivity on the ribosome took place. This indicates that no appreciable exchange of bound C^{14}-phenylalanyl sRNA with free H^3-phenylalanyl sRNA took place under our experimental conditions.

Discussion.—The experiments described above were designed so that C^{14}-phenylalanyl sRNA is not reincorporated into the polypeptide chain after it was once released from ribosomes. The release of once bound C^{14}-phenylalanyl sRNA is negligible as shown in Figure 4. Under the experimental conditions, about 60–70 per cent of C^{14}-phenylalanine of phenylalanyl sRNA initially bound to ribosomes was incorporated into polyphenylalanine. Of the two possible sites for specific binding of phenylalanyl sRNA, site 1 appears to have an easier access to phenylalanyl sRNA than site 2. Since site 1 is on the COOH side of the growing polypeptide chain, the flow of sRNA is from site 1 to site 2. Site 1 has to perform the function of selecting appropriate sRNA according to the direction of messenger RNA. It is therefore reasonable that site 1 has a higher affinity for sRNA than site 2. Recent evidence suggests, however, that site 2 may not be the site for peptidyl sRNA binding.[12] The movement of sRNA from site 2 to the site for peptidyl sRNA binding remains to be elucidated.

In a preliminary experiment, we attempted to determine the site or sites on the 30*S* subunits. In this experiment, the complex of C^{14}-phenylalanyl sRNA, 30*S* subunits, and poly U was made. It was then mixed with 50*S* subunits, C^{12}-phenylalanyl sRNA, GTP plus soluble enzymes, and polyphenylalanine synthesis was allowed to proceed. During the course of this experiment, it was found that a rapid release of phenylalanyl sRNA takes place from 30*S* subunits upon the addition of soluble enzymes and only a small portion of phenylalanine from the initially bound phenylalanyl sRNA is incorporated into polyphenylalanine. Thus, the nature of the sRNA binding site(s) on the 30*S* subunit and their relation to the 70*S* ribosomal sites remain to be elucidated.

Summary.—NH_2-terminal analysis of polyphenylalanine formed from ribosome bound C^{14}-phenylalanyl sRNA suggests that there are two sites for specific binding of phenylalanyl sRNA on 70*S* ribosome. These two sites were named site 1 ($-COOH$ side) and site 2 ($-NH_2$ side) with respect to the growing polypeptide chain. In the presence of excess amounts of ribosomes, sites 1 bind three times more phenylalanyl sRNA than do sites 2.

Note added in proof: Recently, independent evidence for binding of two phenylalanyl sRNA molecules to a ribosome has also been obtained by Nakamoto and his associates (Nakamoto, T., personal communication).

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