Note added in proof: Further evidence was obtained indicating that the ribonucleoside moieties of microsomal RNA are utilized in the synthesis of TMV-RNA.

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[1] Abbreviations: TMV, tobacco mosaic virus; TMV-RNA, tobacco mosaic virus ribonucleic acid; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetate; TCA, trichloroacetic acid; RNAase, ribonuclease; DNAase, deoxyribonuclease.

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*A LEUCINE ACCEPTOR sRNA WITH AMBIGUOUS CODING PROPERTIES IN POLYNUCLEOTIDE-STIMULATED POLYPEPTIDE SYNTHESIS**

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Weisblum, Benzer, and Holley[1] separated two leucine acceptor sRNAs from *E. coli* by countercurrent distribution. They found that the ribosomal incorporation of leucine attached to one of these is stimulated by poly UC, while leucine attached to the other one responds more readily to poly UG and somewhat to poly U. This experiment proved that leucine is carried to the polynucleotide template by two different molecular species of sRNA having different coding properties. It also provided an explanation for the degeneracy observed in coding experiments with leucine.[2, 3] In this paper it is shown that *E. coli* sRNA can be separated by countercurrent distribution into three leucine acceptors. All three have different coding properties: the first responds preferentially to poly UC, the second responds to poly U and copolymers with a high uridylic acid content, including poly UC 5:1, and the third responds preferentially to poly UG.

Materials and Methods.—Separation of three leucine acceptors: *E. coli* B sRNA was prepared by the method of Zubay.[4] The fraction which precipitates between 0.54 and 0.89 vol of isopropanol was passed over a DEAE cellulose column as described by Holley *et al.*[5] Countercurrent distribution (CCD) was done as described by Apgar *et al.*[6] The first five tubes of a 200 tube apparatus (E. C. Apparatus Co., Philadelphia, Pa.) were charged with 170 mg of *E. coli* sRNA. After 200 transfers, the final fractions were combined into 40 sets of 5 tubes each, reisolated as described,[6] and dialyzed overnight against one change of deionized water. The dialyzed fractions were brought to

dryness in a desiccator over sodium hydroxide pellets and sulfuric acid. Each fraction was dissolved in 2 ml of distilled water and the optical density at 260 mμ was determined.

Assay of leucine acceptor activity: A 100,000 × *g* supernatant of an *E. coli* extract, passed through a Sephadex G-25 column to remove amino acids, was used as the source of the leucine-activating enzyme. The reaction mixture for the assay of the leucine acceptor activity of the sRNA fractions contained in a final volume of 0.5 ml: Tris-HCL, pH 7.2, 50 μmoles; $MgCl_2$, 5 μmoles; KCl, 5 μmoles; adenosine triphosphate, K salt (ATP), 0.5 μmoles; 19 C^{12}-amino acids (leucine omitted), 0.2 μmoles each; 0.1 μc of C^{14}-leucine (specific activity: 10 μc/μmole, New England Nuclear Corporation); reduced glutathione (GSH), 2 μmoles; crystalline beef serum albumin, 100 μg; *E. coli* 100,000 × *g* supernatant, 50 μg protein; and 0.05 ml of the sRNA fraction to be tested. The tubes were incubated for 20 min at 35°C. At the end of the incubation 1 mg of carrier *E. coli* ribosomal RNA was added, and the samples were precipitated and washed with salt-ethanol as described by Berg *et al.*[7] The precipitate was dissolved in dilute ammonia, dried on planchets, and counted in a low background gas-flow counter (Nuclear-Chicago Corporation), with a C^{14}-counting efficiency of 20%.

Preparation of C^{14}-leucyl-sRNA: To remove the sRNA present in the *E. coli* extract used as the source for the leucine-activating enzyme, 10 ml of the 100,000 × *g* supernatant was passed through a 1 × 5 cm column of DEAE cellulose (Serva Entwicklungslabor, Heidelberg), previously equilibrated with 0.1 *M* Tris HCl buffer pH 7.5 containing 0.006 *M* mercaptoethanol.[8,9] The effluent and 10 ml of wash were collected and dialyzed overnight against standard buffer containing 0.01 *M* Tris-HCl pH 7.4, 0.01 *M* Mg Cl_2, 0.06 *M* KCl, 0.006 *M* β-mercaptoethanol. The extract was stored frozen. The reaction mixture contained in a final volume of 2 ml: Tris-HCl pH 7.2 200 μmoles; Mg Cl_2, 20 μmoles; KCl, 20 μmoles; ATP, 5 μmoles; GSH, 8 μmoles; 19 C^{12}-amino acids (leucine omitted), 0.4 μmole each; *E. coli* extract, treated as above, 100 μg protein; C^{14}-leucine (specific activity 246 μc/μmole, New England Nuclear Corporation), 0.04 μmole; sRNA from fraction 14 (Fig. 1, peak 1), 2.6 mg; from fraction 32 (Fig. 1, peak 2), 0.6 mg; from fraction 36 (Fig. 1, peak 3), 0.5 mg, respectively. The three tubes were incubated for 25 min at 35°C, the reaction was stopped by adding 2 ml of redistilled phenol, the tubes were shaken for 10 min at room temperature, and then centrifuged in the cold. The water layer was made 2% with potassium acetate and 0.02 molar with C^{12}-leucine. Then the sRNA was precipitated with 2 vol of ethanol at −20°C; the precipitate was washed once with cold salt-ethanol (see above), dissolved in 2% potassium acetate containing 0.02 *M* leucine, and reprecipitated with 2 vol of ethanol at −20°C. After two more washings with salt-ethanol, the precipitate was dried in a vacuum and the sRNA dissolved in 1 ml of deionized water. In the case of peak 1, 2.4 mg of aminoacyl-sRNA were obtained containing 0.47 × 10^6 cpm per mg sRNA, in the case of peak 2, 0.54 mg sRNA containing 1.3 × 10^6 cpm per mg sRNA, and in the case of peak 3, 0.46 mg sRNA containing 1.04 × 10^6 cpm per mg sRNA.

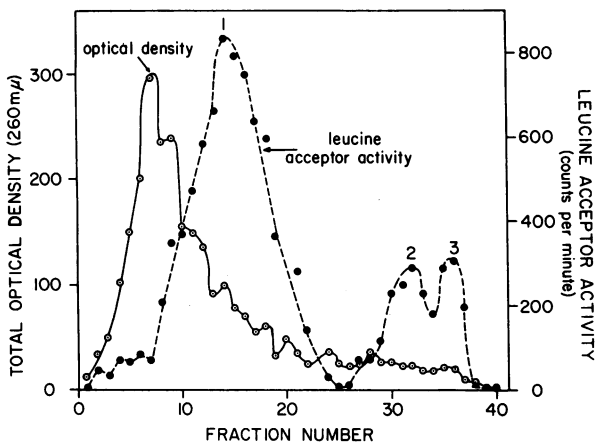

FIG. 1.—Separation of three leucine acceptors by a 200-transfer countercurrent distribution of *E. coli* sRNA (conditions, see text). Each fraction was dissolved in 2 ml of water. The leucine acceptor activity refers to the C^{14}-leucine incorporated into 0.05 ml of each fraction.

Unfractionated *E. coli* sRNA was charged with C^{14}-leucine (specific activity 10 μc/μmole) and reisolated by the method described.[10] It contained 4,000 cpm per mg sRNA. sRNA charged with a mixture of 19 nonradioactive amino acids was prepared in the same way, except that the C^{14}-leucine was omitted.

Preparation of E. coli extract for leucine transfer: Preincubated 30,000 × *g* supernatant of *E. coli* extract (iS-30) was prepared from frozen bacterial paste essentially as described by Nirenberg and Matthaei,[11] except that the cells were broken in a French pressure cell, the 30,000 × *g* supernatant of the extract was preincubated for 45 min at 35°C in standard buffer without any further additions, and, instead of dialysis, the extract was passed over a Sephadex G-50 column. The preparation used had an OD_{260} of 240 per ml. When tested for activity as described,[11] 250-fold stimulation of incorporation of free C^{14}-phenylalanine was produced by 15 μg of poly U.

Transfer of C^{14}-leucine bound to sRNA into polypeptide: The reaction mixture contained in a final volume of 1 ml: Tris-HCl pH 7.6, 100 μmoles; $MgCl_2$, 15 μmoles; K Cl, 50 μmoles; guanosine triphosphate, 0.05 μmole; phosphoenolpyruvate K salt, 7 μmoles; pyruvate kinase, 20 μg; β-mercaptoethanol, 6 μmoles; 19 C^{12}-amino acids, 0.2 μmoles each; C^{12}-leucine, 2 μmoles; C^{12}-aminoacyl-sRNA (leucine omitted), 0.2 mg; C^{14}-leucyl-sRNA as stated in the legends of Figures 2, 3, 4, and 5; 5 μg poly U, poly UG 5:1, poly UA 5:1, poly UC 5:1, or poly UC 1:1 as indicated; iS-30, 0.1 ml.

The mixture was incubated at 35°C. At the times indicated, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid (TCA). The RNA was hydrolyzed for 15 min in a boiling water bath and the precipitate was collected and washed with 5% TCA, containing carrier leucine, on a Millipore filter. The filters were glued on planchets, dried, and counted. The amount of amino acid transferred was proportional to the aminoacyl-sRNA added.

Synthetic polyribonucleotides: Poly UC (input ratio 1:1) and poly UG (input ratio 5:1) were kindly prepared for us by Dr. R. F. Beers. Poly UA (input ratio 5:1) and poly UC (input ratio 5:1) were kindly donated by Dr. B. Weisblum. Poly U was purchased from the Miles Chemical Company, Clifton, New Jersey.

Results.—Separation of three leucine acceptors: The results of a 200-transfer countercurrent distribution of *E. coli* sRNA are shown in Figure 1. The recovery of ultraviolet-absorbing material was 75 per cent. The assay of leucine acceptor activity shows three peaks, one broad peak with the maximum at fraction 14, which is clearly separated from a double peak, moving almost with the front, with maxima at fractions 32 and 36. Samples taken from these fractions will subsequently be referred to as peak 1, peak 2, and peak 3 sRNA.

Coding properties of peak 1, peak 2, and peak 3 sRNA: Aliquots of fractions 14, 32, and 36 were charged with C^{14}-leucine, and their response to different polymers was determined. Poly UA 5:1 and poly UC 5:1 were tested in addition to the polymers used by Weisblum *et al.*,[1] poly UC 1:1, poly UG 5:1, and poly U.

The kinetics of transfer of C^{14}-leucine from peak 1 sRNA in response to these 5 polymers is shown in Figure 2. Both poly UC 1:1 and poly UC 5:1 are active; poly UC 5:1 produces the highest level of stimulation. Although the response of peak 1 sRNA to poly UA and poly UG is small, it nevertheless is significant because it was obtained repeatedly. Thus, the coding properties of our peak 1 sRNA are similar to the coding properties reported by Weisblum *et al.*[1] for their peak 1 sRNA.

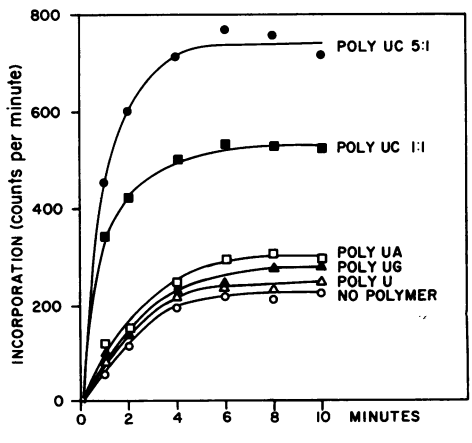

FIG. 2.—Polynucleotide-dependent incorporation of leucine bound to peak 1 sRNA. 0.013 mg C^{14}-leucyl peak 1 sRNA were added, containing 6,000 cpm (conditions as described in *Methods*). The input ratio of nucleotides in the synthesis of the copolymers was 5:1 unless noted otherwise.

The response of peak 2 sRNA to the polymers is shown in Figure 3. In this case all polymers with a high uridylic acid content stimulated the transfer of C^{14}-leucine; poly UG and poly U were the most active. It should be noted that the UC copolymer, which was found inactive with the peak 2 sRNA by Weisblum *et al.*,[1] was the poly UC 1:1. In agreement with their result, poly UC 1:1 had only a small but significant effect. The poly UC 5:1, on the other hand, was almost as active as the other copolymers containing a high proportion of uridylic acid. It is concluded therefore that the coding properties of this leucine acceptor sRNA are very similar to the coding properties that have been reported for the phenylalanine acceptor sRNA.[3, 11, 12]

Figure 4 shows the response of peak 3 sRNA to the 5 polymers. Poly UG has by far the greatest effect, whereas poly UA, poly UC 5:1, and poly U have dropped considerably.

The relatively high level of stimulation by poly UG in the case of peak 2 sRNA, and the still significant stimulation by poly UA, poly U, and poly UC 5:1, in the case of peak 3 sRNA, is probably due to the incomplete separation of peaks 2 and 3 sRNA. It is very likely that the peak 2 sRNA of Weisblum *et al.*[1] contained a mixture of our peak 2 and peak 3 sRNA.

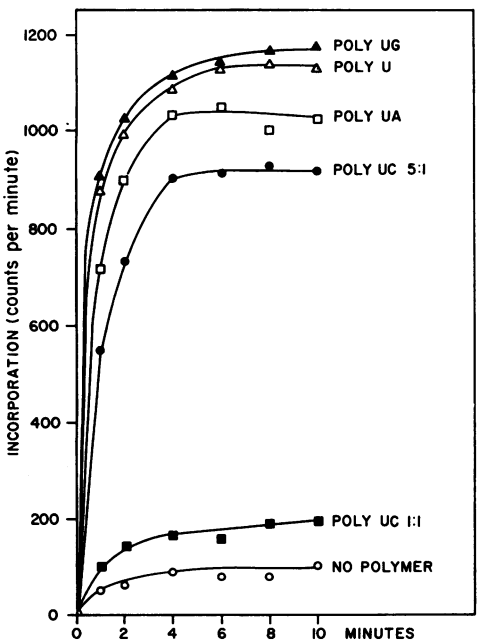

FIG. 3.—Polynucleotide-dependent incorporation of leucine bound to peak 2 sRNA. 0.006 mg C^{14}-leucyl peak 2 sRNA were added, containing 7,900 cpm (conditions as described in *Methods* and in Fig. 2).

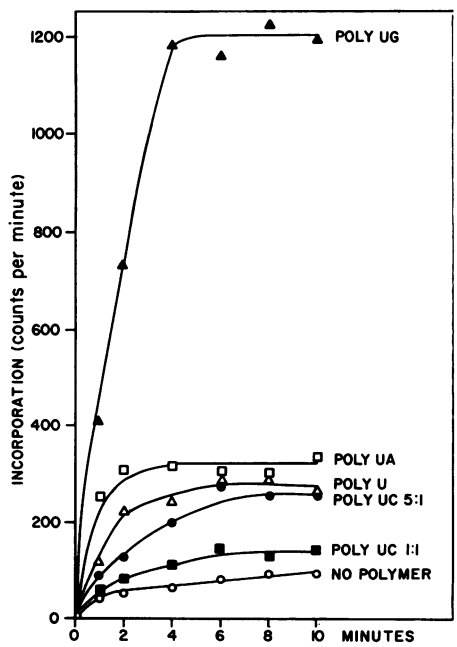

FIG. 4.—Polynucleotide-dependent incorporation of leucine bound to peak 3 sRNA. 0.005 mg C^{14}-leucyl peak 3 sRNA were added, containing 5,200 cpm (conditions as described in *Methods* and in Fig. 2).

The pattern of transfer of C^{14}-leucine charged to unfractionated sRNA in response to the various polymers is shown in Figure 5. The relative abundance of peak 1 sRNA versus peak 2 and peak 3 sRNA in the unfractionated mixture is reflected by the relatively large effect of poly UC 1:1 and also of poly UC 5:1. It should be

kept in mind that both peak 1 sRNA and peak 2 sRNA respond to poly UC 5:1.

Discussion.—The results support the conclusion by Weisblum *et al.*[1] that degeneracy in the case of leucine can be accounted for by the different coding properties of different acceptors. As has been pointed out by the same authors, two kinds of degeneracy are possible. In the first kind more than one coding unit specifies one amino acid; in the second kind one coding unit specifies more than one amino acid, and in this case the coding unit is said to be ambiguous. The existence of ambiguity in the amino acid code was suggested by the observation that both phenylalanine and leucine are incorporated by *E. coli* ribosomes in response to poly U.[1, 13, 14] The experiment described in this paper shows that leucine is carried to the template by a separate leucine acceptor which has ambiguous coding properties.

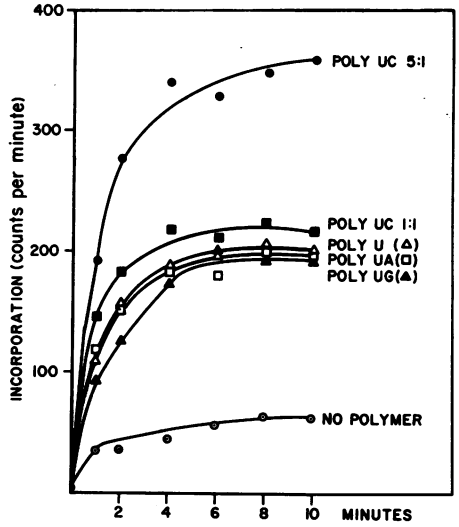

FIG. 5.—Polynucleotide-dependent incorporation of leucine bound to unfractionated *E. coli* sRNA. 1.0 mg of C^{14}-leucyl-sRNA were added, containing 4,000 cpm. The specific activity of the C^{14}-leucine was lower (10 μc/μmole) than that used in Figs. 2, 3, and 4 (conditions as described in *Methods* and in Fig. 2).

Whether the results obtained with the artificial polymers also apply to the synthesis of natural proteins is currently being tested by transferring leucine bound to these three acceptors into the leucine positions of hemoglobin in a ribosomal system from rabbit reticulocytes,[10] and into the leucine positions of the coat protein of the MS_2-bacteriophage utilizing the system described by Nathans *et al.*[15]

Summary.—*E. coli* contains three leucine acceptor sRNAs separable by countercurrent distribution. Transfer into polypeptide of leucine attached to the first one is stimulated preferentially by poly UC, leucine attached to the second one is transferred in response to polynucleotides containing a high proportion of uridylic acid including poly U, and leucine attached to the third acceptor is incorporated preferentially in response to poly UG. These results provide an explanation for the ambiguity in coding for leucine observed in polynucleotide-dependent polypeptide synthesis.

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*PROTEIN SYNTHESIS BY RETICULOCYTE RIBOSOMES, I. INHIBITION OF POLYURIDYLIC ACID-INDUCED RIBOSOMAL PROTEIN SYNTHESIS BY CHLORAMPHENICOL**

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Chloramphenicol is known to inhibit microbial protein synthesis both in intact cells[1-4] and in cell-free systems.[5, 6] Similar inhibition of protein synthesis has not, however, been demonstrable in analogous mammalian systems.[7-9] Matthaei and Nirenberg observed inhibition of protein synthesis by *E. coli* ribosomes with 0.3 μmoles of chloramphenicol per ml reaction mixture.[5] However, von Ehrenstein and Lipmann were unable to obtain an appreciable inhibition in mammalian cell-free systems with 0.01 molar amounts of chloramphenicol.[7] Similarly, inhibition of amino acid incorporation into intact reticulocytes *in vitro* has been noted only with amounts of chloramphenicol which greatly exceed pharmacologic concentrations.[9]

Although mammalian ribosomes do not appear to be affected by chloramphenicol, there is considerable evidence that chloramphenicol can exert deleterious effects in man. Cells of the hematopoietic system appear to be particularly susceptible as manifested by the occasional development of anemia, leukopenia, thrombocytopenia, or aplastic anemia during chloramphenicol therapy.[10, 11] Although the mechanism of toxicity is unknown, certain clinical observations suggest that chloramphenicol is most likely to affect primitive or immature cells during a stimulatory or maturation phase. Thus, Saidi *et al.*[12] observed that patients with pernicious anemia in relapse who were receiving chloramphenicol at the time when B_{12} therapy was initiated, failed to manifest the reticulocyte response seen as a result of this type of therapy. Furthermore, the characteristic reticulocyte response did not occur in these patients until after chloramphenicol was discontinued. In such patients normoblastic maturation of the megaloblastic marrow occurred despite the inhibition of reticulocytosis, indicating that purine and pyrimidine biosynthesis was unaffected. This is analogous to the sequence of events in bacteria where chloramphenicol inhibits protein synthesis without inhibiting DNA or RNA synthesis.[1]

These observations suggest that megaloblasts may be uniquely susceptible to chloramphenicol inhibition, whereas cells at a later stage of maturation remain un-