CODING PROPERTIES OF E. COLI LEUCYL-sRNA's CHARGED WITH HOMOLOGOUS OR YEAST ACTIVATING ENZYMES

BY THOMAS PETER BENNETT, JACK GOLDSTEIN, AND FRITZ LIPMANN

THE ROCKEFELLER INSTITUTE
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The redundancy, or degeneracy as it is generally called, of amino acid coding has been deduced from various observations. Holley and his group, by applying countercurrent distribution to the mixture of sRNA's, obtained two or more distinct sRNA peaks for the same amino acid. The use of polynucleotides of fixed composition, introduced by Nirenberg and his group, and by Ochoa and collaborators, for probing polypeptide synthesis from activated amino acids, has indicated that the same amino acid can respond with polypeptide formation to different polynucleotides.

A pronounced redundancy was found for leucine, which showed incorporation into polypeptide chains in a system stimulated by poly UC, poly UG, poly UA, or poly U. The recently observed specific coding of one leucine-specific countercurrent peak by poly UC, and the other by poly UG, as well as less pronouncedly by poly U, amplifies these observations by indicating the possibility of a coding difference between the two leucine sRNA fractions.

The matching of the activating enzyme of one species with the sRNA from another species has indicated only a partial overlap and qualitative as well as quantitative differences in the sRNA.

Since a number of observations indicate universality of the code, a differential response to activating enzymes has been interpreted to mean that the affinity of amino acid-specific sRNA, when matching the activating enzyme, may not be due to the coding nucleotide combination. Such a conclusion, however, may need revision, as will be seen from the experiments to be reported here. In expanding on the type of experiments first made by Berg, it now appears that a crude E. coli sRNA preparation, that is partially charged with leucine by yeast enzyme, responds almost exclusively to poly UC in a similar manner to the countercurrent peak I-sRNA in the experiments of Weisblum et al., while the remainder appears to be coded preferentially by poly UG. Experiments on the question of code universality will also be reported.

Methods.—Soluble RNA from E. coli B was prepared by the phenol procedure described by von Ehrenstein and Lipmann.

Enzyme preparations: E. coli B cells (log phase) were ruptured after the method of Berg. The extract was centrifuged twice at 30,000 × g for 30 min in the 9-in. rotor of the Lourdes centrifuge, and at 105,000 × g for 2 hr in the No. 40 rotor of the Spinco. The 105,000 × g supernatant was stored frozen; when thawed, it was passed, just before use, through a Sephadex G-25 column that had been equilibrated with 0.01 M phosphate-0.005 M mercaptoethanol buffer pH 7.0.

National bakers' yeast was stirred with liquid nitrogen for several minutes. The nitrogen was permitted to evaporate and the ruptured yeast cells were immediately stored at −20°. For 2 min, frozen yeast was blended in a Virtis homogenizer with glass beads and 0.01 M Tris-0.005 M mercaptoethanol pH 7.5. The extract was centrifuged at 30,000 × g for 30 min, then at 105,000 × g for 2 hr. The 105,000 × g supernatant was passed through Sephadex G-25 just before use.

Attachment of C14-leucine to sRNA: The procedure of von Ehrenstein and Lipmann was used to attach C14-leucine to either yeast or E. coli sRNA by enzymes from yeast or E. coli extracts;
50 nmol of C14-L-leucine (105 μC/μM) were used per milligram of sRNA charged. Incubation was at 35° for 20 min, unless otherwise stated.

Attachment of C14-leucine to E. coli sRNA previously charged with C14-leucine by yeast enzyme: The reaction mixture for attaching C14-leucine to sRNA contained, in a total volume of 1.5 ml: 300 μM Tris HCl pH 7.2, 8.0 μM MgCl2, 8.0 μM GSH, 4.0 μM ATP, 5.0 μM phosphoenolpyruvate (PEP) Na salt, 10 μg of PEP kinase, 100 μM C14-leucine, 3 mg of sRNA, and 0.30 mg protein of 105,000 × g yeast extract. After incubation for 20 min at 35°, the charged sRNA was isolated by the phenol procedure. The product was dialyzed at pH 5.8 for 7 hr at 0° against deionized water.

The reaction mixture for attaching C14-leucine to the dialyzed product contained, in a total volume of 1.5 ml: 300 μM cacodylate-K pH 6.6, 8.0 μM MgCl2, 8.0 μM GSH, 4.0 μM ATP, 5.0 μM PEP, 10 μg of PEP kinase, 2 μg of myokinase, 2 μg of pyrophosphatase, 2 μC C14-leucine (105 μC/μM), 2 μM amino acid mixture minus leucine, E. coli enzyme extract (0.20 mg protein), and the dialyzed product from above. After incubation for 5 min at 35°, the charged sRNA was isolated by the phenol procedure.

Poly nucleotide-stimulated ribosomal incorporation of C14-leucine bound to sRNA: S-30 was prepared according to the procedure of Matthaei and Nirenberg. The protein concentration of the product was 12 mg/ml.

Essentially the method of Weisblum et al.7 was used to establish the code properties of leucyl-sRNA preparations. The reaction mixture contained, in a total volume of 0.25 ml: 15 μM Tris HCl pH 7.5, 1.2 μM PEP, 8 μg of PEP kinase, 7.5 μM KCl, 2 μM GSH, 5 mM C14-leucine, 0.1 mg of C14-aminoacyl-sRNA omitting leucine, 10 μg of poly UG, UC, or U, as indicated in the figures, 50 μl of S-30, and C14-Leucyl-sRNA. After incubation, the reaction was stopped by addition of 7% trichloroacetic acid (TCA), and the resulting precipitate was digested, washed on a Millipore filter, and counted in a windowless gas-flow counter.

f2 RNA-stimulated ribosomal incorporation of C14-leucine bound to sRNA: The reaction mixture for f2 RNA-stimulated incorporation of leucine from leucyl-sRNA was essentially the same as above, except that cacodylate-K buffer at pH 6.8 was substituted for Tris-HCl pH 7.5, and 0.25 μM ATP was added in a 0.25 ml total incubation volume. f2 RNA was added to the reaction mixture in amounts indicated in the tables and figures. The reaction was terminated and incorporation of C14-leucine into TCA-insoluble precipitate was determined as above.

Separation of two leucine acceptor RNA's: sRNA from E. coli B was prepared by phenol extraction and passage over DEAE-cellulose, as described by Doctor et al.1 Two leucine acceptor fractions were isolated by countercurrent distribution after 500 transfers in a solvent system modified after Apagar et al.13 The pooled fractions used for the experiments to be presented were taken at positions 100 tubes apart from each other in the train of the countercurrent machine. The procedures and methods used are to be published.

Materials.—ATP and GTP were products of Pabst Laboratories, Milwaukee, Wis. PEP Na salt and pyruvate were purchased from California Corp. for Biochemical Research. C14-L-leucine (105 μC/μM) and other C14-amino acids were obtained from Schwarz BioResearch, Inc. Poly UG (5:1) and poly UC (1:1) were kindly donated by Drs. J. Allende and Z. Kucan, respectively. Poly U was purchased from Miles Laboratory, Clifton, N. J., or was prepared in this laboratory. Yeast sRNA was purchased from General Biochemicals, Inc., Chagrin Falls, Ohio. f2 RNA was the generous gift of Dr. Norton Zinder.

Results.—Transfer of amino acids to E. coli sRNA by yeast enzyme: Berg et al.,8 Rendi and Ochoa,18 and Benzer and Weisblum14 have studied the species-specific attachment to sRNA of a variety of amino acids in response to heterologous activating enzyme preparations from E. coli, yeast, or animal tissue. In the following experiments, comparison was restricted to E. coli sRNA and enzymes from yeast and E. coli. The yeast enzyme used here differs from that used by Rendi and Ochoa in its derivation from a different yeast strain and in the extraction procedure. This may explain the negative response of their preparation with leucine in contrast to the differential response reported here. Our experiments confirmed the observations of Benzer and Weisblum that yeast enzyme preparations connect lysine but
not tyrosine to *E. coli* sRNA. Leucine, alanine, serine, isoleucine, and aspartic acid were also found to be attached by yeast enzyme, but phenylalanine and histidine were not (Table 1).

Our bakers' yeast preparation charged *E. coli* sRNA with leucine, although to only two thirds the extent charged by *E. coli* enzyme. This differential effect, which corresponds qualitatively to the observations of Berg *et al.* with methionine, was studied in greater detail. Figure 1 shows that if increasing amounts of *E. coli* sRNA (sRNAcₐ) are charged with leucine by either yeast or *E. coli* enzymes, the proportion of charge remains the same. It appears from the experiments of Table 2 that sRNAc₀ is charged maximally within 15 min by the *E. coli* enzyme or the yeast enzyme, although only 65 per cent as much leucine is linked to sRNAc₀ by the yeast enzyme. If, however, incubation with yeast enzyme for 15 min was followed by incubation with *E. coli* enzyme for a further 15 min, the attachment of leucine was equal to that obtained with *E. coli* enzyme alone, or with a mixture of *E. coli* and yeast enzymes.

The coding characteristics of differentially labeled leucyl-sRNAcₒ: Weisblum *et al.* had observed that sRNAcₒ appeared to consist of a mixture, which we propose to designate sRNAc₀Leu¹ and sRNAc₀Leu², that responded to either a poly UC- or a poly UG-template. In view of their observation and of our finding only a partial charging of sRNAc₀ by yeast enzyme, we decided to test whether the distinction made by the yeast enzyme might sort out one or the other sRNA's from the mixture.

**TABLE 1**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>sRNAcₐ (cpm)</th>
<th>sRNAc₀ (cpm)</th>
<th>sRNAcₒ (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>1409</td>
<td>1350</td>
<td>2080</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1840</td>
<td>1020</td>
<td>2590</td>
</tr>
<tr>
<td>Lysine</td>
<td>800</td>
<td>320</td>
<td>430</td>
</tr>
<tr>
<td>Serine</td>
<td>560</td>
<td>234</td>
<td>407</td>
</tr>
<tr>
<td>Alanine</td>
<td>580</td>
<td>620</td>
<td>870</td>
</tr>
<tr>
<td>Histidine</td>
<td>400</td>
<td>10</td>
<td>230</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>700</td>
<td>60</td>
<td>485</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>800</td>
<td>350</td>
<td>490</td>
</tr>
</tbody>
</table>

The reaction mixture contained, in a total volume of 0.75 ml: 100 mM Tris HCl pH 7.4, 16 mM MgCl₂, 8 mM ATP, 16 mM GSH, 0.1 μg C¹⁴-amino acid (10 μc/μg), 300 μg of yeast enzyme, 75 μg of *E. coli* enzyme, and 0.14 μg of sRNAc₀ or sRNAcₐ, as indicated. After incubation at 36° for 20 min, the reaction was stopped by addition of cold 7% TCA. The precipitate was washed on a Millipore filter with cold TCA, dried, and counted.
Fig. 2.—Polymer-dependent incorporation of C\textsuperscript{14}-leucine bound to unfractionated \textit{E. coli} sRNA by \textit{E. coli} enzyme. Reaction mixture and procedure as in Methods. 0.13 mg of C\textsuperscript{14}-leucyl-sRNA\textsubscript{co} containing 9,750 cpm was added per point. The specific activity of the leucine was 105 µc/µM.

![Graph](image)

Table 2

<table>
<thead>
<tr>
<th>Enzyme additions</th>
<th>Total incubation (min)</th>
<th>Incorporation C\textsuperscript{14}-leucine (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. coli} enzyme</td>
<td>15</td>
<td>0.27</td>
</tr>
<tr>
<td>\textit{E. coli} enzyme; yeast enzyme after 15 min</td>
<td>30</td>
<td>0.28</td>
</tr>
<tr>
<td>Yeast enzyme</td>
<td>15</td>
<td>0.17</td>
</tr>
<tr>
<td>\textit{E. coli} enzyme; yeast enzyme after 15 min</td>
<td>30</td>
<td>0.28</td>
</tr>
<tr>
<td>Yeast + \textit{E. coli} enzyme</td>
<td>30</td>
<td>0.25</td>
</tr>
</tbody>
</table>

The reaction mixture and conditions were the same as those in Table 1, except that each tube contained 0.2 µM of 19 amino acids (leucine omitted) and 0.16 mg of sRNA\textsubscript{co}.

of sRNA\textsubscript{co}\textsuperscript{Leu}. In the experiments presented in Figures 2 and 3, polypeptide formation is compared in the poly UC and UG systems. As shown in Figure 2, sRNA\textsubscript{co}\textsuperscript{Leu} charged with leucine by \textit{E. coli} enzyme responds equally as well to poly UG as to poly UC. It appears, however, (Fig. 3) that the yeast enzyme-charged leucyl-sRNA\textsubscript{co}\textsuperscript{Leu} is utilized much more readily for polypeptide formation with the UC polymer than with the UG, following essentially the response pattern of the countercurrent distribution peak I fraction, sRNA\textsubscript{co}\textsuperscript{Leu} I.

When the yeast enzyme selectively charges 65 per cent of leucyl-sRNA having UC coding properties, the 35 per cent of remaining sRNA\textsubscript{co}\textsuperscript{Leu} should code with poly UG. To prove this, sRNA\textsubscript{co} was incubated with C\textsuperscript{12}-leucine and yeast enzyme, and the C\textsuperscript{12}-leucyl-sRNA was isolated by the phenol procedure. After dialysis, this preparation was tested with yeast enzyme, and no appreciable further charge with C\textsuperscript{14}-leucine was detected. The preparation was then charged with C\textsuperscript{14}-leucine by \textit{E. coli} enzyme. To minimize nonenzymatic deacylation of the C\textsuperscript{12}-leucyl-sRNA,
this second incubation was carried out at pH 6.6; furthermore, pyrophosphatase and myokinase were added to counteract possibilities of enzymatic reversion.

The coding properties of the sRNA remaining after preliminary incubation with C$^{14}$-leucine are presented in Figure 4. It appears that the sRNA$^{14}_{Leu}$ left after charging with yeast enzyme is analogous to the second countercurrent fraction sRNA$^{14}_{Leu}$ II, and almost exclusively responds to poly UG. The yeast enzyme thus reacts only, or at least preferably, with sRNA$^{14}_{Leu}$ I, but leaves sRNA$^{14}_{Leu}$ II largely uncharged.

Separation of the two leucyl-sRNA fractions and their coding characteristics: Following Apgar et al.,$^{12}$ we have obtained two distinct leucyl-sRNA fractions by countercurrent distribution. This has made it possible to confirm the findings of Weisblum et al.$^{7}$ of an apparently physical basis for code degeneracy, and to show unequivocally that yeast enzyme attaches leucine only to the E. coli sRNA with UC coding properties. Coding properties of peak I and peak II were examined after charging with C$^{14}$-leucine. In confirmation of the earlier study,$^{7}$ peak I was most active with poly UC, whereas peak II was most effective for leucine polymerization on poly UG. Polymer-dependent incorporation of leucine bound to peak I was identical to that shown for unfractionated sRNA$^{14}_{Leu}$ charged by yeast enzyme (Fig. 3). When transfer efficiency as high as 50 to 70 per cent was obtained, however, a significant response to the other polymer was observed, i.e., poly UG for peak I or poly UC for peak II. Also, poly U induced measurable response with both peak I and peak II fractions. Since the two countercurrent fractions were 100 tubes apart, gross contamination is excluded. The possibility, however, that the polynucleotides used in these experiments have small amounts of bases as contaminant, is being examined further. From present tests it would seem that contamination, if any, would be too small to explain the observed effects. It was observed, however, that five different poly U samples gave varied responses with leucine in contrast to a constant response with phenylalanine. This fact, as well as the recent finding of Jones and Nirenberg$^{15}$ of a competition between phenylalanine and leucine, suggests caution in interpreting the response of different leucyl-sRNA preparations in the poly U system.

The curves in Figure 3 suggest that yeast enzyme charges only peak I sRNA$^{14}_{Leu}$. A direct test of this is presented in Table 3. Although both peaks can be charged by E. coli enzyme, only peak I is charged with leucine by yeast enzyme. During our studies we learned that a similar effect has been observed by Keller with rat liver enzyme$^{16}$ where leucine could be attached to peak I sRNA$^{14}_{Leu}$ but not to peak II.

The coding characteristics of yeast leucyl-sRNA (sRNA$^{14}_{Leu}$): When the coding
Properties of leucyl-sRNA\textsubscript{Leu} charged with yeast enzyme were determined by the method used for sRNA\textsubscript{Co} (Fig. 2), a strong response to poly UG and an only slightly smaller response to poly UC (or poly U) were observed. Such a redundant response might have been expected from the observations of Doctor et al.\textsuperscript{1} who reported the separation of two leucine-responsive sRNA fractions in yeast. Although sRNA\textsubscript{Leu} is partially compatible with yeast enzyme, \textit{E. coli} enzyme did not charge sRNA\textsubscript{Leu}, although a variety of conditions were tried. Yeast enzyme with sRNA\textsubscript{Leu} thus charges both the poly UC- as well as the poly UG-responsive sRNA\textsubscript{Leu} fractions, but with sRNA\textsubscript{Co} this same enzyme preparation charges only, or rather preferentially, sRNA\textsubscript{Leu}\textsuperscript{1}.

Tests with a natural messenger RNA: Recently, Nathans et al.\textsuperscript{17} showed the formation in vitro of f2 virus coat protein by using f2 RNA as messenger in an \textit{E. coli} ribosomal system. We have found a rather good incorporation of C\textsuperscript{14}-leucine from unfractionated leucyl-sRNA\textsubscript{Co} in the f2 RNA-stimulated system described in Methods. It can be seen from Table 4 that our system is particularly effective when cacodylate buffer and a pH of about 7 are used. In experiments, presented in Figure 5, C\textsuperscript{14}-leucyl-sRNA prepared from either sRNA\textsubscript{Co} or sRNA\textsubscript{Co}\textsuperscript{1} were incubated with f2 RNA as messenger in the system used above; the results indicate that the two varieties of leucyl-sRNA give incorporation with comparable rates into protein. The relative amounts incorporated in these experiments are about one quarter from the C\textsuperscript{14}-leucyl-sRNA\textsubscript{Co} and one fifth from sRNA\textsubscript{Co}\textsuperscript{1}. Analogous experiments with leucyl-sRNA\textsubscript{Leu} and yeast-charged leucyl-sRNA\textsubscript{Co} also showed incorporation on f2 RNA-primed ribosomes.

The amount of protein available from these experiments was not sufficient to carry out a detailed analysis. The fact that sizeable incorporation of C\textsuperscript{14}-leucine occurs from either of the two leucyl-sRNA's, presumably the only active C\textsuperscript{14}-leucines available, make it likely that both leucyl-sRNA's can furnish leucine here for polypeptide synthesis.

Comments.—Comparable results to Berg et al.\textsuperscript{8} have now been obtained with sRNA\textsubscript{Co} for leucine charging, two thirds responding to yeast enzyme and \textit{E. coli},
the differential response, showing that the yeast enzyme-responsive sRNA\textsubscript{Leu} is the peak I sRNA, the sRNA\textsubscript{Co}\textsubscript{1} of Holley. The remainder is the second countercurrent peak, which could be charged subsequently and separately with \textit{E. coli} enzyme. Furthermore, yeast enzyme-charged sRNA\textsubscript{Leu} will discharge preferentially on poly UC, while the remainder, analogous to sRNA\textsubscript{Co}\textsubscript{II}, discharges preferentially on poly UG.

The sorting out by yeast enzyme of the poly UC-responsive sRNA\textsubscript{Co} is surprising, since yeast sRNA\textsubscript{Leu} appears to include both the poly UC- and the poly UG-responsive sRNA's as indicated by yeast enzyme-charged leucyl-sRNA\textsubscript{Leu} discharging with both polymers. The fact that in the present study the response of leucyl-sRNA\textsubscript{Leu}\textsuperscript{1} to poly UC and of sRNA\textsubscript{Co}\textsubscript{II} to poly UG was preferential rather than exclusive, and both responded to poly U, may still be explained through our use of slightly contaminated polynucleotides. But if that should not be the case, we seem to be in need of a better understanding of the enzymatic events during discharging of the leucyl-sRNA's.

The confirmation and extension of the heterogeneity of the two sRNA's demands all the more reconciliation with the essentially confirmed indications of universality of coding at the level of the template, the mRNA. Furthermore, it appears that yeast enzyme-charged sRNA\textsubscript{Leu} discharges well on f2 RNA messenger, supporting the coding crossover between yeast and \textit{E. coli} leucyl-sRNA.

Preliminary experiments indicate that both of the \textit{E. coli} leucyl-sRNA's appear to feed into polypeptide bond formation with very similar kinetics when f2 RNA serves as messenger. Experiments in collaboration with Drs. Zinder and Schwartz are in progress to define the type of protein formed and the position of the leucine derived from the different leucyl-sRNA's. Only then will it be possible to compare the coding properties of the two sRNA fractions in the polynucleotide and natural mRNA systems.

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6 Weisblum, B. S. Benzer, and R. W. Holley, these \textit{PROCEEDINGS}, 48, 1449 (1962).
BIOPHYSICAL CHARACTERISTICS OF THE RNA-CONTAINING BACTERIAL VIRUS R17

BY M. D. ENGER, E. A. STUBBS, S. MITRA, AND PAUL KAESBERG

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN

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The discovery of a bacteriophage containing RNA has stimulated considerable interest in the isolation and characterization of other RNA-containing bacterial viruses. Among those which have subsequently been isolated is R17.

The receipt of a sample of R17 and of its host, *E. coli* K-12 (Hfr, methionine−), from Dr. Angus Graham of the Wistar Institute has enabled us to investigate physical and chemical properties of the virus and of its components. R17 appears to be eminently suitable for studies of virus structure and synthesis. It is stable, can be grown in considerable quantity, and is readily purified. It can be degraded to yield protein subunits and stable, high-molecular weight RNA.

This report concerns intact R17. Investigations of the viral protein and RNA will be reported elsewhere.

**Methods.**—**Growth and purification:** R17 was grown on *E. coli* K-12 (Hfr, methionine−) in shake-flask cultures to virus concentrations of 10^{12} to 10^{13} plaque-forming units per ml. Cells and debris were removed by centrifugation. The pH of the supernatant liquid of the lysate was slowly lowered to 4 by the addition of 20% acetic acid, and the resulting precipitate was removed by centrifugation and discarded. Three hundred and fifty gm of ammonium sulfate were added for each liter of liquid. The material which precipitated was spun down, taken up in 0.05 M Na phosphate buffer, pH 7.0, and subjected to 3 cycles of centrifugation of 15 min at 15,000 rpm (discard sediment), and 150 min at 30,000 rpm (resuspend sediment) in the No. 30 rotor of a Spinco Model L preparative centrifuge. All procedures were performed at 4°–10°C. The final sediment, resuspended in buffer, gave a single symmetrical peak in the analytical centrifuge and contained 30–80% of the plaque-forming units present in the original lysate. Its homogeneity will be documented in the next section.

**Sedimentation:** Sedimentation velocity analyses were made in a Spinco Model E analytical centrifuge at 35,600 rpm. The temperature was regulated at 20°C throughout all runs. The observed sedimentation coefficients were corrected to standard conditions and are designated $s_{20,w}$.

**Diffusion:** Diffusion was analysed in a Spinco Model H electrophoresis-diffusion apparatus with Rayleigh optics. Creeth's procedure for calculation was used. The diffusion coefficient was corrected to standard conditions and is designated $D_{20,w}$.

**Ultraviolet absorption:** Absorption spectra were determined with a Cary Model 15 spectrophotometer. Corrections for light scattering are small and were made by extrapolation from the neighboring nonabsorbing region of the spectrum. For most of the biophysical measurements reported here, concentration was determined by ultraviolet absorption at 260 m.$\mu$. 

10 Maxwell, E. S., these **Proceedings**, 48, 1639 (1962).
16 Keller, E., personal communication.