RNA Codewords and Protein Synthesis

The Effect of Trinucleotides upon the Binding of sRNA to Ribosomes

Marshall Nirenberg and Philip Leder

Although many properties of the RNA code and protein synthesis have been clarified with the use of synthetic polynucleotides containing randomly ordered bases, a more comprehensive understanding of certain aspects of the code clearly requires investigation with nucleic acid templates of demonstrated structure. Since oligonucleotides of known base sequence are readily prepared and characterized, we have tried, in many ways, to use defined oligonucleotide fractions for studies relating to the RNA code. In this article we describe a simple, direct method which should provide a general method for determining the genetic function of triplets of known sequence. The system is based upon interactions between ribosomes, aminoacyl sRNA (1), and mRNA which occur during the process of codeword recognition, prior to peptide-bond formation.

The binding of sRNA to ribosomes has been observed in many studies (2, 3); however, this interaction is not fully understood. An exchangeable binding of sRNA to ribosomes was reported by Cannon, Krug, and Gilbert (4). However, the addition of polyU induced, with specificity, Phe-sRNA binding to ribosomes, as demonstrated in the laboratories of Schweet (5-7) and Lipmann (8), by Kaji and Kaji (9, 10), and by Spyrides (11). Binding was reported to be dependent upon GTP (6, 7) and the first transfer enzyme (5-7), but not upon peptide-bond synthesis. However, the mechanism of binding and the possibility of a prior, nonenzymatic binding of aminoacyl sRNA induced by mRNA have not been clarified. The second transfer enzyme was shown to be required for peptide bond formation (6, 7).

To determine the minimum chain length of mRNA required for codeword recognition and to test the ability of chemically defined oligonucleotides to induce C"-aminoacyl-sRNA binding to ribosomes, we have devised a rapid method of detecting this interaction and have found that trinucleotides are active as templates.

Methods

Preparation, purification, and characterization of oligonucleotides. To obtain oligonucleotides with different terminal groups, polyA, polyU, and polyC (12) were digested as follows: (i) Oligonucleotides with 5'-terminal phosphate; 100 mg of polynucleotide were incubated at 37°C for 18 hours in a 28-ml reaction mixture containing 29mM tris, pH 7.2; 0.18mM MgCl2; 0.23mM 2-mercaptoethanol; 8.0 mg crystalline bovine albumin and 0.5 mg pork liver nuclease (13). (ii) Oligonucleotides with 3'(2')-terminal phosphate; 100 mg of polynucleotide were incubated at 37°C for 24 hours in 20 ml of 7.0M NH4OH. (iii) Oligonucleotides without terminal phosphate; Oligonucleotides with terminal phosphate were treated with Escherichia coli alkaline phosphatase (14) free of diesterase activity as described by Hoppel et al. (15).

Oligonucleotide fractions were separated on Whatman 3 MM paper by chromatography with solvent A (H2O:n-propanol:NH4, 35:55:10; by volume) for 36 hours (fractions with terminal phosphate) or for 18 hours (fractions without terminal phosphate). This procedure fractionates oligonucleotides containing less than eight nucleotide residues according to chain length. Oligonucleotides were eluted with H2O and further purified on Whatman 3 MM paper by electrophoresis at pH 2.7 (0.05M ammonium formate, 80 v/cm for 15 to 30 minutes).

After elution the purity of each fraction was estimated by subjecting 2.5 A492 units of each to paper chromatography (Whatman 54 paper) both with solvent A and with solvent B (40 g ammonium sulfate dissolved in 100 ml 0.1M sodium phosphate, pH 7.0). In addition 3.0 A492 units of each oligonucleotide were subjected to chromatography on Whatman DE 81 (DEAE) paper with solvent C (0.1M ammonium formate), and 3.0 A492 units with solvent D (0.3M ammonium formate). The four chromatographic systems described separate homologous series of oligonucleotides according to chain length. Contaminating oligonucleotides were present in amounts greater than 2 percent could be detected. Several preparations of each oligonucleotide were used during the course of this study. In almost all preparations, no contaminants were detected. The following preparations, specified in legends of figures or tables when used, contained contaminants in the proportions indicated: No. 591, (Ap)4 [(Ap)3, 11 percent]; No. 599, (pA)5 [(pA)4, 37 percent]; No. 610, (pU)2 [(pU)1, 14 percent]; No. 613 (pU)3 [(pU)2, 14 percent]; No. 617, (Up)4 [(Up)3, 12 percent].

Base composition and position of terminal phosphate were determined by digesting 2.0 A492 units of each oligonucleotide preparation with 3.5 X 105 units of T5 ribonuclease (16) in 0.1M NH4HCO3 at 37°C for 2.5 hours. The nucleotide and nucleoside products were separated by electrophoresis at pH 2.7 and identified by their mobilities and ultraviolet spectra at pH 2.0. Oligonucleotides with 5'-terminal phosphate yielded the appropriate 5'-pentose diphosphate, 3'-pentose monophosphate, and nucleoside. From the ratio of these compounds, the average chain length of the parent oligonucleotide was calculated. Since oligonucleotides with 3'-terminal phosphate yielded only the appropriate 3'-nucleoside monophosphate (confirming its structure), terminal and total inorganic phosphate was determined (15, 17) in order to estimate the average chain length of each.

Oligo-d(T) and oligo-d(PA) fractions were prepared and characterized by B. F. C. Clark as described previously (18). The UpUpUp with 3'-
Table 1. Characteristics of the system. Complete reactions contained the components described in the text, 15 μmole uridylic acid residues in polyU, and 20.6 μmole C-4-Phe-sRNA (2050 count/min, 0.714 A₄⁰⁰ units). Incubation was at 0°C for 60 minutes. Deacetylated sRNA was added either at zero time or after 50 minutes of incubation, as indicated.

<table>
<thead>
<tr>
<th>Modifications</th>
<th>C₄-Phe-sRNA bound to ribosomes (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>5.99</td>
</tr>
<tr>
<td>-PolyU</td>
<td>0.12</td>
</tr>
<tr>
<td>-Ribosomes</td>
<td>0</td>
</tr>
<tr>
<td>+sRNA (deacetylated) at 50 min</td>
<td>0.09</td>
</tr>
<tr>
<td>0.500 A₄⁰⁰ units</td>
<td>5.69</td>
</tr>
<tr>
<td>2.500 A₄⁰⁰ units</td>
<td>5.36</td>
</tr>
<tr>
<td>+sRNA (deacetylated) at zero time</td>
<td>4.49</td>
</tr>
<tr>
<td>0.500 A₄⁰⁰ units</td>
<td>2.08</td>
</tr>
<tr>
<td>2.500 A₄⁰⁰ units</td>
<td>2.08</td>
</tr>
</tbody>
</table>

Terminal phosphate only was prepared and characterized by M. Bernfield (19).

Assay of ribosomal bound C-4-aminoacyl-sRNA. Each 50-μl reaction mixture contained: 0.1M tris-acetate, pH 7.2; 0.02M magnesium acetate; 0.05M KCl; 2.0 A₂₆₀ units of ribosomes. Each 50-μl reaction mixture contained the components described in the text, prepared and characterized by M. Bernfield (19). Terminal phosphate only was prepared in a stainless steel holder in a 0°C water bath. A cellulose nitrate filter (HA pore size) in a stainless steel holder was washed under gentle suction with portions of buffer at 0°C to 3°C. A cellulose nitrate filter (HA pore size) in a stainless steel holder was washed under gentle suction with portions of buffer at 0°C to 3°C. The diluted reaction mixture was immediately poured on the filter under suction and washed to remove unbound Cα-aminoacyl sRNA with three, 3-ml portions of buffer at 0°C to 3°C. Ribosomes and bound sRNA remained on the filter. Since reaction mixtures are not deproteinized, it is important to dilute and wash the ribosomes immediately after incubation, to use cold buffer, and to allow relatively little air to be pulled through the filter during the washing procedure. The filter was removed from the holder, glued with rubber cement to a disposable planchette, and dried. Radioactivity was determined in a thin-window, gas-flow counter (20) with a Cα-counting efficiency of 65 percent.

Preparation of sRNA. Except where noted E. coli B sRNA (23), was used. Uniformly labeled C-1-L-phenylalanine, C-14-L-lysine, and C-14-L-leucine with specific radioactivities of 250, 305, and 160 μc/μmole, respectively, were obtained commercially (24). The E. coli W 3100 sRNA was prepared as described by Zubay (25) from cells grown to late log phase in 0.9 percent Difco nutrient broth, containing 1 percent glucose. The C-α-amino acyl-sRNA was prepared by modifications of meth-

Table 2. Polynucleotide specificity. Reaction mixtures containing C-14-Phe- and C-14-Lys-sRNA were incubated for 60 minutes at 0°C; mixtures containing C-14-Pro-sRNA were incubated for 20 minutes at 24°C. In addition to the components described in the text, reaction mixtures contained, in a final volume of 50 μl, the specific polynucleotide and C-14-aminoacyl-sRNA (14.7 μmole C-14-Phe-sRNA, 2015 count/min, 0.960 A₄⁰⁰ units; 16.5 μmole C-14-Lys-sRNA, 1845 count/min, 0.530 A₄⁰⁰ units; 30 μmole C-14-Pro-sRNA, 2750 count/min, 1.570 A₄⁰⁰ units).

<table>
<thead>
<tr>
<th>Polynucleotide (μmole base residues)</th>
<th>C-14-Aminoacyl-sRNA bound to ribosomes (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.19, 0.99, 0.25</td>
</tr>
<tr>
<td>PolyU, 25</td>
<td>6.00, .67, .15</td>
</tr>
<tr>
<td>PolyA, 16</td>
<td>0.22, 4.35, .17</td>
</tr>
<tr>
<td>PolyC, 19</td>
<td>0.21, 0.72, .80</td>
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</tbody>
</table>

Fig. 1 (left). Effect of polyU upon the rate of C-14-Phe-sRNA binding to ribosomes at 0°C, 24°C, and 37°C. Each point represents a 50-μl reaction mixture incubated for the time and at the temperature indicated. Reaction mixtures contain the components described under Methods; 9.65 μmole of C-14-Phe-sRNA (1180 count/min, 0.380 A₄⁰⁰); and polyU, 25 μmole base residues, where specified. Fig. 2 (right). The effect of polyA and polyC upon the rate of C-14-Lys- and C-14-Pro-sRNA binding to ribosomes, respectively. Each point represents a 50-μl reaction mixture as described under Methods. The reactions specified contained 27.5 μmole C-14-Lys-sRNA (3080 count/min, 0.880 A₄⁰⁰ units) and polyA, 25 μmole base residues, or 11.8 μmole C-14-Pro-sRNA (2660 count/min, 0.905 A₄⁰⁰ units) and polyC, 25 μmole base residues. The temperature and the time of incubation are shown in the figure.
ods described previously (26). Unless otherwise specified, sRNA was acylated with one C4- amino acid plus 19 C4- amino acids. The formation of C4- aminoacyl sRNA was catalyzed by the supernatant solution obtained by centrifugation of E. coli (W-3100) extracts at 100,000g.

**Elution and characterization of C4- phenylalanine product bound to ribosomes.** Reaction mixtures (0.5 ml) incubated at 24°C for 10 minutes with C4-Phe-sRNA and polyU were washed on cellulose nitrate filters in the usual manner. The ribosomal bound C4-product was eluted from filters by washing with 0.01M tris-acetate, pH 7.2; 10^2 M magnesium acetate; and 0.05M KCl at 0°C.

The C4-product eluted from ribosomes was precipitated in 10 percent TCA at 3°C in the presence of 200 µg of bovine serum albumin. Specified samples were heated in 10 percent TCA at 90°C to 95°C for 20 minutes and then were chilled. Precipitates were washed on filters with 5 percent TCA at 3°C.

Aminoacyl sRNA was deacylated in 0.1M ammonium carbonate solution adjusted with NH4OH to pH 10.2 to 10.5 by incubation at 37°C for 60 minutes.

Digestions with ribonuclease were performed by incubating 0.4-ml portions (each containing 1500 count/min precipitable by 10 percent TCA at 3°C) with and without 10 µg of purified pancreatic ribonuclease A (purified chromatographically) (27) at 37°C for 15 minutes.

**Results and Discussion**

**Assay of ribosomal bound C4-aminoacyl sRNA.** The assay is based upon the retention of ribosomes and C4- aminoacyl sRNA bound to ribosomes by cellulose nitrate filters. After unbound C4-aminoacyl sRNA is removed on cellulose nitrate filters. After unbound C4-aminoacyl sRNA was catalyzed by the supernatant solution obtained by centrifugation of E. coli (W-3100) extracts at 100,000g.

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**Results and Discussion**

**Assay of ribosomal bound C4-aminoacyl sRNA.** The assay is based upon the retention of ribosomes and C4- aminoacyl sRNA bound to ribosomes by cellulose nitrate filters. After unbound C4-aminoacyl sRNA is removed by washing with buffered salts solution, as already described, the radioactivity remaining on the filter is determined. Thirty reaction mixtures can be washed per hour easily. The sensitivity of the assay is limited primarily by the specific radioactivity of the aminoacyl-sRNA used. With sRNA which has accepted a C4-amino acid of specific radioactivity 100 to 300 µCi/µmole, the binding to ribosomes of 0.2 µmole of C4-aminoacyl sRNA readily can be detected. A filter 25 mm in diameter with a pore size of 0.45 µ retains up to 1200 µg of E. coli ribosomes. The use of larger filters or columns packed with cellulose nitrate may be useful for preparative procedures.

The retention of ribosomes by cellulose nitrate filters may be the result of absorption rather than of filtration, for filters with pores 100 times larger than E. coli 70S ribosomes can be used. The rapidity of this assay, compared to others which depend upon the centrifugation of ribosomes, has greatly simplified this study.

The data of Table 1 show that little C4-Phe-sRNA was retained on filters after incubation with ribosomes in the absence of polyU. Incubation in the presence of both polyU and ribosomes resulted in marked retention of C4- Phe-sRNA by the filter. Ribosomes, polyU, and Mg2+ were required for retention of C4-Phe-sRNA. Spyrides (28) and Conway (29) have reported that polyU-directed binding of Phe-sRNA to ribosomes is dependent upon K+ or NH4+.

The addition of deacylated sRNA to reactions shortly before incubation was terminated, after C4-Phe-sRNA binding had ceased, had little effect upon ribosomal bound C4-Phe-sRNA. The bound C4-Phe-sRNA fraction apparently is not readily exchangeable.

In contrast, the addition of deacylated sRNA at the start of incubation inhibited C4-Phe-sRNA binding. In other experiments, the extent of inhibition was found to be affected by the ratio of deacylated to acylated sRNA.

Deacylated sRNA added near the end of incubation often reduces background binding without polynucleotide and may afford a way of differentiating between exchangeable and nonexchangeable binding. It should be noted that the presence of a polynucleotide which is not recognized by a C4- aminoacyl sRNA (for example, polyA and C4-Phe-sRNA) also reduces background sRNA binding, perhaps by saturating ribosomal sites with specified nonexchangeable sRNA.

**Characteristics of binding.** The assay was validated further by demonstrating that the binding of sRNA to ribosomes was directed with specificity by different polynucleotides. As shown in Table 2, polyU, polyA and polyC specifically directed the binding to ribosomes of C4-Phe-, C4-Lys-, and C4-Pro-sRNA, respectively. These data agree well with specificity data obtained with a sucrose-density centrifugation assay, reported by Nakamoto et al. (8) and Kaji and Kaji (9, 10) (also compare 5-7, 11) and with data on their specificity for directing amino acid incorporation into protein (30, 31).

The rate of binding of Phe-sRNA to ribosomes at 0°, 24° and 37°C, in the presence and absence of polyU, is shown in Fig. 1. During incubation at each temperature polyU markedly stimulated C4-Phe-sRNA binding; how-
ever, the rate of binding increased as the temperature of incubation was raised. Although polyU induced C\textsuperscript{14}-Phe-sRNA binding at 0\°C, maximum binding was not observed after 60 minutes of incubation. Maximum binding concentrations of C\textsuperscript{14}-Phe-sRNA, 50 minutes of incubation at 24\°C and after 6 minutes at 37\°C. In this experiment, equal amounts of C\textsuperscript{14}-Phe-sRNA were bound at 24\°C and 37\°C. In other experiments, with limiting concentrations of C\textsuperscript{14}-Phe-sRNA, 50 to 98 percent of the C\textsuperscript{14}-Phe-sRNA was induced to bind to ribosomes by polyU. Kaji and Kaji have suggested the possible utility of this system for the purification of sRNA species (9).

In the absence of polyU, relatively little C\textsuperscript{14}-Phe-sRNA associated with ribosomes. Such binding may be due to endogenous mRNA on ribosomes or in sRNA preparations. Alternatively, this binding may be nonspecific, possibly similar to that described by Cannon, Krug, and Gilbert (4).

The effect of polyA and polyC upon the rates of C\textsuperscript{14}-Lys- and C\textsuperscript{14}-Pro-sRNA binding to ribosomes is shown in Fig. 2. Maximum stimulation of C\textsuperscript{14}-Lys-sRNA binding by polyA, and of C\textsuperscript{14}-Pro-sRNA binding by polyC, occurred after 10 minutes of incubation at 24\°C and 27\°C, respectively. In this experiment, C\textsuperscript{14}-Lys-sRNA binding observed in the absence of polyA was higher than that found in experiments with other sRNA preparations.

The relation between polyU or polyA concentration and the amount of C\textsuperscript{14}-Phe- or C\textsuperscript{14}-Lys-sRNA bound to ribosomes is shown in Fig. 3. Binding of sRNA was proportional to polynucleotide concentration in both cases.

In experiments not presented here, the effect of pH upon sRNA binding was studied. PolyU directed C\textsuperscript{14}-Phe-
Table 3. Oligonucleotide specificity. Reaction mixtures containing either C¹⁴-Phe-, C¹⁴-Lys-, or C¹⁴-Pro-sRNA, and oligonucleotide were incubated at 24°C for 20 minutes. Components of reaction mixtures are described in the legend accompanying Table 2. The numbers in parentheses are millimicromoles base residues.

<table>
<thead>
<tr>
<th>Oligonucleotide (mμmoles base residues)</th>
<th>C¹⁴-Phe-sRNA bound to ribosomes (mμmol)</th>
<th>C¹⁴-Lys-sRNA bound to ribosomes (mμmol)</th>
<th>C¹⁴-Pro-sRNA bound to ribosomes (mμmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.34</td>
<td>0.80</td>
<td>0.24</td>
</tr>
<tr>
<td>pUpUpU (10)</td>
<td>1.56</td>
<td>0.56</td>
<td>0.20</td>
</tr>
<tr>
<td>pApApA (7)</td>
<td>0.20</td>
<td>6.13</td>
<td>0.18</td>
</tr>
<tr>
<td>pCpCcC (8)</td>
<td>0.30</td>
<td>0.60</td>
<td>0.73</td>
</tr>
</tbody>
</table>

The relation between ribosome concentration and the amount of bound C¹⁴-Phe-sRNA is illustrated in Fig. 4. C¹⁴-Phe-sRNA binding was stimulated markedly by polyU and was proportional to the ribosome concentration, within the range of 0 to 1.0 A⁶⁶⁶ units of ribosomes. The number of 70S E. coli ribosomes and ribosomal-bound C¹⁴-Phe-sRNA molecules can be estimated from such data; however, various factors, such as the inhibitory effects of deacylated sRNA (see above) and mRNA terminal phosphate (described below) undoubtedly reduce the accuracy of such calculations. However, in the presence of polyU approximately 4.0 μmol of C¹⁴-Phe-sRNA became bound to 23.2 μ mole of 70S ribosomes (4); therefore, the C¹⁴-Phe-sRNA ribosome ratio was 1:5.8. Arlinghaus et al. (7) and Warner and Rich (32) recently reported two binding sites for each ribosome for sRNA. One site is thought to hold the nascent polypeptide chain to the ribosome; the other, to bind the next aminoacyl-sRNA molecule specified by mRNA.

The relation between ribosome concentration and the amount of bound C¹⁴-aminoacyl-sRNA to ribosomes, at 0°, 24°, and 37°C, is shown in Fig. 5. The C¹⁴-Phe-sRNA binding was stimulated by pUpUpU at each temperature; however, binding was maximum in reactions incubated at 24°C for 20 to 30 minutes. These results demonstrate that a trinucleotide can direct C¹⁴-aminoacyl-sRNA binding to ribosomes and suggest a general method of great simplicity for determining the genetic function of other trinucleotide sequences.

The effect of oligonucleotides on the binding of C¹⁴-aminoacyl sRNA to ribosomes. OligoU preparations of different chain length were prepared, and their effect on C¹⁴-Phe-sRNA binding to ribosomes was determined. The effect of the trinucleotide, pUpUpU, upon C¹⁴-Phe-sRNA binding to ribosomes, at 0°, 24°, and 37°C, is shown in Table 4. The C¹⁴-Phe-sRNA binding was stimulated by pUpUpU at each temperature; however, binding was maximum in reactions incubated at 24°C for 20 to 30 minutes. These results demonstrate that a trinucleotide can direct C¹⁴-aminoacyl-sRNA binding to ribosomes and suggest a general method of great simplicity for determining the genetic function of other trinucleotide sequences.

Table 4. Template activity of oligodeoxynucleotides. The components of each 50 μl reaction mixture are presented in the text. In addition, each reaction mixture in Expt. 1 contained 9.65 μmoles of C¹⁴-Phe-sRNA (1370 count/min, 0.380 A⁶⁶⁶ units); and in Expt. 2, 16.5 μmoles C¹⁴-Lys-sRNA (1845 count/min, 0.530 A⁶⁶⁶ units) and the oligonucleotides specified. Mixtures were incubated at 24°C for 10 minutes.

<table>
<thead>
<tr>
<th>Oligonucleotide (mμmol)</th>
<th>C¹⁴-Aminoacyl-sRNA bound to ribosomes (mμmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.29</td>
</tr>
<tr>
<td>1.00 pUpUpU</td>
<td>1.29</td>
</tr>
<tr>
<td>3.33 pUpUpU</td>
<td>2.40</td>
</tr>
<tr>
<td>6.67 pUpUpU</td>
<td>2.90</td>
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<tr>
<td>3.33 oligo d(pT)₉</td>
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</tr>
<tr>
<td>6.67 oligo d(pT)₉</td>
<td>0.39</td>
</tr>
<tr>
<td>10.00 oligo d(pT)₁₃</td>
<td>0.40</td>
</tr>
<tr>
<td>1.67 oligo d(pT)₁₃</td>
<td>0.31</td>
</tr>
<tr>
<td>2.50 oligo d(pT)₁₃</td>
<td>0.44</td>
</tr>
</tbody>
</table>

The data of Table 4 indicate that the 2'-hydroxyl of RNA codewords may be necessary for codeword recognition. Oligodeoxynucleotides such as oligo-d(pT)₉ and oligo-d(pA)₉ apparently were inactive as templates. In additional experiments not presented here, the effects of time and temperature of incubation (0°, 24°, 37°, and 43°C), template concentration, and chain length were studied. No template activity was found.

The template activities of pUpU, pUpUpU and polyU at different concentrations of Mg⁺⁺ are shown in Fig. 6. Both tri- and polyU induced maximal binding at approximately 0.03M Mg⁺⁺ (0.02 to 0.03M in other experiments). Although the dinucleotide, pU, pUpU, stimulated binding slightly, it is not known whether the activity of pUpU indicates partial recognition of a triplet codeword as previously suggested (35). At 0.02M Mg⁺⁺, the concentration used throughout our study, little binding of C¹⁴-Phe-sRNA was found in the absence of polyU. However, at higher Mg⁺⁺ concentrations, Phe-sRNA binding in the absence of template RNA increased.
whereas binding induced by tri- and polyU, decreased. These data suggest that certain ribosomal binding sites become saturated with sRNA at Mg⁺ concentrations greater than 0.03M, but are not saturated at lower concentrations.

Leucine-polyU ambiguity. Since polyU is known to direct some leucine incorporation into protein in cell-free systems (36), especially at high Mg⁺ concentrations (37), the effect of polyU upon the binding of C⁴-Leu-sRNA was determined (Fig. 7). In the absence of polyU, background binding saturated at 0.02M Mg⁺. The addition of polyU clearly stimulated the binding of C⁴-Leu-sRNA. It is possible that the Mg⁺-dependent leucine-polyU ambiguity occurs before peptide-bond synthesis.

As shown in Table 5, C⁴-Leu-sRNA binding was stimulated with specificity by polyU, but not by the trinucleotide, pUpUpU, polyA, or polyC at 0.07M Mg⁺. In additional experiments, pUpU had no effect upon the binding of C⁴-Leu-sRNA at other Mg⁺ concentrations. These data suggest that pUpUpU may be recognized by aminoacyl sRNA with greater specificity than polyU is recognized.

**Effect of oligonucleotide chain length, concentration, and terminal phosphate.** In Fig. 8 the template activity of oligoU preparations differing in chain length and position of terminal phosphate are compared at different oligoU concentrations. The activity of oligoU with 5'-terminal phosphate is shown in Fig. 8A; preparations with 2'-(3')-terminal phosphate in Fig. 8B; and preparations without terminal phosphate, in Fig. 8C. As shown in Fig. 8A, the dinucleotide with 5'-terminal phosphate, pUpU, had little template activity, whereas the trinucleotide, pUpUpU, markedly stimulated C⁴-Phe-sRNA binding. This ob-

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**Fig. 8.** A, B, and C. Relation between template activity and oligoU chain-length, concentration and end-group. The activities of oligoU with 5'-terminal phosphate are shown in Fig. 8A; with 2'-(3')-terminal phosphate in Fig. 8B; and without terminal phosphate in Fig. 8C. Symbols represent oligoU chain-lengths as follows: ○ 2; △ 3; □ 4; ■ 5; ● 6; ▲ 12. Each reaction mixture contained, in a volume of 50 µl, the components specified in the Methods section; oligoU preparations and concentrations specified; and 10.8 µmole C⁴-Phe-sRNA (1880 count/min, 0.714 A₆₆₀). Oligonucleotide preparations (pU)₁₀, No. 610; (pU)₁₂, No. 613; (Up), No. 617; with the contaminants specified under Methods were used in these experiments. Incubations were at 24°C for 30 minutes.

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**Fig. 9.** A, B, and C. Relation between template activity and oligoA concentration, chain-length, and end-group. The activity of oligoA with 5'-terminal phosphate is shown in Fig. 9A; preparation without terminal phosphate in Fig. 9C. The symbols indicate the chain-length of oligoA as follows: ○ 2; △ 3; □ 4; ■ 5; ● 6. Each 50 µl reaction mixture contained the components specified under methods; 15.9 µmole of C⁴-Lys-sRNA (1780 count/min, 0.562 A₆₆₀ units); and the oligoA preparation specified. Incubation was at 24°C for 20 minutes. Oligonucleotide preparations (pA)₉, No. 599 and (Ap)₄, No. 591, containing the contaminants specified under Methods, were used in this experiment.
observed provides direct experimental support for a triplet code for phenyl-
alanine and is in full accord with earlier genetic and biochemical studies (30, 38).

In addition, the data demonstrate that the template activity of pUpUpU equals that of the corresponding tetra-, penta-, and hexanucleotides.

In striking contrast to these results, the trinucleotide, UpUpUp, with 2'(3')-terminal phosphate, induced little or no binding of C\textsuperscript{14}-Phe-sRNA to ribosomes (Fig. 8B). The template activity of the tetra- and pentauridylic acid fractions with 2'(3')-terminal phosphate also were markedly reduced when compared to similar fractions with 5'-terminal phosphate.

As shown in Fig. 8C, UpUpU and UpUpUpU, without terminal phosphate, induced C\textsuperscript{14}-Phe-sRNA binding, but less actively than pUpUpU. The template activity of the pentamer, UpUpUpUpU, was almost equal to that of pUpUpU.

Since the sensitivity of an oligonucleotide to digestion by a nuclease often is influenced by terminal phosphate, the relative stability of pUpUpU, UpUpUp, and UpUpU incubated with ribosomes at 37°C for 60 minutes was estimated by recovering mono- and oligonucleotides from reaction mixtures and separating them by paper chromatography. In each case, the expected trinucleotide was the only component found after incubation. Hydrolysis of oligoU was not observed.

The template activities of oligoA fractions with different end groups are shown in Fig. 9, A, B, and C. The results were similar to those obtained with oligoU; however, pApApA induced maximum C\textsuperscript{14}-Lys-sRNA binding at one-fifth the oligonucleotide concentration required previously (compare with Fig. 8A). The hexamer with 3'-terminal phosphate induced as much C\textsuperscript{14}-Lys-sRNA binding to ribosomes as the trimer with 5'-terminal phosphate, pApApA. When reaction mixtures were incubated at 0°C (Fig. 10) the difference between the template activities of ApApAp and pApApA was more marked than when incubations were at 24°C.

Since each oligonucleotide preparation with 2'(3')-terminal phosphate is a mixture of molecules, some chains terminating with 2'-phosphate and others with 3'-phosphate, a trinucleotide, UpUpUp, with 3'-terminal phosphate only, was prepared and found to be ineffective as a template for C\textsuperscript{14}-Phe-sRNA.

**Attachment of ribosomes to mRNA.**

It is not known whether ribosomes attach to 5'-ends, 3'-ends, or internal positions of mRNA. The template activity of trinucleotides indicates that (i) ribosomes can attach to terminal codewords of mRNA, (ii) terminal codewords are capable of specifying the first and the last amino acids to be incorporated into protein, and (iii) the attachment of a ribosome to only the terminal triplet of mRNA may provide the minimum stability necessary for codeword recognition, and possibly for the initiation of protein synthesis.

The demonstration that terminal and internal codeword phosphates strongly influence the codeword recognition process indicates that phosphate may take part in the binding of codewords to ribosomes. Watson has suggested interaction between phosphate of mRNA and amino groups of ribosomes, because 30S ribosomes treated with formaldehyde were found by Moore and Asano to bind less polyU than did ribosomes not so treated (39).

Although terminal codeword phosphate is not required for the recognition of a codeword on a ribosome, the observation that the template activity of trinucleotides with 5'-terminal phosphate equals that of tetra-, penta-, and hexanucleotides, even at limiting concentrations, suggests that 5'-terminal codewords may attach to sites on ribosomes where codewords are recognized, in correct phase to be read. A preferential, phased recognition of either terminal codeword by ribosomes would provide a simple mechanism for selecting the polarity of reading, the first word to be read, and the phase of reading. Since 5'-terminal codewords of mRNA most actively induce sRNA binding, such codewords would appear to serve these functions best. Although the polarity with which mRNA is read may be from the 5'-wards to the 3'-terminal codeword, further work is necessary to clarify this point. The opposite polarity has been suggested (45).

We have reported (40) that trinucleotides can be used in this system to determine the base sequence of codewords and have shown that the sequence of the valine RNA codeword is GpUpU. Codewords are recognized with polarity in this system, for GpUpU induced C\textsuperscript{14}-Val-sRNA binding to ribosomes, whereas UpUpG did not.
Genetic evidence suggests that certain mutations result in the conversion of readable into nonreadable codewords, that is, sense-nonsense interchanges (41). The addition of terminal phosphate to a 3'-terminal codeword similarly changes a readable into a nonreadable codeword and resembles a sense-nonsense interchange. Two additional mechanisms for converting readable into nonreadable words have been found in cell-free systems; that is, an increase in secondary structure (37, 42) and specific base methylations (37). It should be noted that each type of sense-nonsense interchange involves a modification of codewords rather than modification of a component required for codeword recognition.

It is possible that the synthesis of certain proteins may be regulated in vivo by sense-nonsense interchanges involving either modification of a codeword or, as proposed by Ames and Hartman (43), modification of sRNA, that is, codeword recognition. It seems probable that terminal codewords may have special functions in addition to directing amino acids into protein. For example, in mRNA they may specify (i) attachment and detachment of ribosomes, (ii) the first codeword to be read, (iii) the phase of reading, and (iv) the sensitivity of the message to degradation by exonucleases. Similarly, terminal DNA codewords may influence the rate with which DNA is copied by DNA or RNA polymerase. Experimental observations support this possibility, for DNA without terminal phosphate has been found to serve as a template for DNA polymerase, whereas DNA with 3'-terminal phosphate has no template activity (44).

Terminal words with 3'-phosphate may be members of a larger class of DNA and mRNA nonsense words, with substituted 5', 3', or 2'-hydroxyl groups. Many enzymes have been described which catalyze the transfer of nucleotides, amino acids, methyl groups, carbohydrates, and other molecules, to or from mononucleotides or terminal ribose or deoxyribose of nucleic acids. Such enzymes may recognize terminal bases or conformations of nucleic acids and catalyze group transfer reactions with great specificity.

The data of Figs. 8 to 10, and the chemical and biological considerations described, suggest that codeword modification may serve a regulatory or operator function. Modification of codewords, at both terminal and internal positions, may regulate the reading of DNA or RNA by converting a readable word into one read incorrectly or not read. It should be noted that both 3'- and 5'-terminal codewords could serve, in different ways, as operator words.

The capacity of trinucleotides to direct the binding of sRNA to ribosomes and the ease with which the process can be assayed should provide a general method of great simplicity for studying the base sequence and genetic functions of each triplet codeword. In addition, this method should permit the detailed study of interactions between codewords, sRNA, and ribosomes during the codeword recognition process.
Summary

A rapid, sensitive method is described for measuring C4-aminoacyl-sRNA interactions with ribosomes which are specifically induced by the appropriate RNA codewords prior to peptide-bond formation. Properties of the codeword recognition process and the minimum oligonucleotide chain length required to induce such interactions are presented. The trinucleotides, pUpUpU, ApApA, and PcPcC, but not dinucleotides, specifically direct the binding to ribosomes of phenylalanine-, lysine-, and proline-sRNA, respectively.

Since 5'-terminal, 3'-terminal, and internal codewords differ in chemical structure, three corresponding classes of codewords are proposed. The recognition of each class in this system is described. The template efficiency of missense or nonsense codewords, is suggested as a possible regulatory mechanism in protein synthesis.

References and Notes

1. The following abbreviations and symbols are used: Phe, phenylalanine; Leu, leucine; Lys, lysine; Pro, proline; Val, valine; polyU, polyuridylic acid; polyC, polycytidylic acid; polyA, polyanadenylic acid; TCA, trichloroacetic acid; d(pT), deoxythymidylic acid; d(pA), deoxyadenylic acid; sRNA, transfer RNA; mRNA, messenger RNA; DEAE, diethylaminoethyl cellulose; PPOPOP, 1,4-bis-(5'-phenylxazolyl) benzene; PEP, phosphoenolpyruvate; Tm, melting temperature; 49°, at 260 mµ.

2. For mono- and oligonucleotides of specific structure, the p to the left of a terminal phosphate indicates a 5'-terminal phosphate; the p to the right, a 2' (3')-terminal phosphate. Internal phosphates of oligonucleotides are 3' (5')-linkages.


15. L. A. Heppel, personal communication.
