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# 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 amino-acyl 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<sup>14</sup>-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 MgCl<sub>2</sub>; 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 NH<sub>4</sub>OH. (iii) *Oligonucleotides without terminal phosphate*; Oligonucleotides with terminal phosphate were treated with *Escherichia coli* alkaline phosphatase (14) free of diesterase activity as described by Hepel *et al.* (15).

Oligonucleotide fractions were separated on Whatman 3 MM paper by chromatography with solvent A (H<sub>2</sub>O:n-propanol:NH<sub>3</sub>, 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 nucleo-

tide residues according to chain length. Oligonucleotides were eluted with H<sub>2</sub>O 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 A<sup>260</sup> 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 A<sup>260</sup> units of each oligonucleotide were subjected to chromatography on Whatman DE 81 (DEAE) paper with solvent C (0.1M ammonium formate), and 3.0 A<sup>260</sup> units with solvent D (0.3M ammonium formate). The four chromatographic systems described separate homologous series of oligonucleotides according to chain length. Contaminating oligonucleotides 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)<sub>3</sub> [(Ap)<sub>3</sub>, 11 percent]; No. 599, (pA)<sub>3</sub> [(pA)<sub>3</sub>, 37 percent]; No. 610, (pU)<sub>3</sub> [(pU)<sub>3</sub>, 10 percent], No. 613 (pU)<sub>3</sub> [(pU)<sub>3</sub>, 14 percent]; No. 617, (Up)<sub>3</sub> [(Up)<sub>3</sub>, 12 percent].

Base composition and position of terminal phosphate were determined by digesting 2.0 A<sup>260</sup> units of each oligonucleotide preparation with 3.5 × 10<sup>9</sup> units of T<sub>2</sub> ribonuclease (16) in 0.1M NH<sub>4</sub>HCO<sub>3</sub> 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'-3'(2')-nucleoside diphosphate, 3'(2')-nucleoside monophosphate, and nucleoside. From the ratio of these compounds, the average chain length of the parent oligonucleotide was calculated. Since oligonucleotides with 3'(2')-terminal phosphate yielded only the appropriate 3'(2')-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(pT) and oligo-d(pA) fractions were prepared and characterized by B. F. C. Clark as described previously (18). The UpUpUp with 3'-

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Table 1. Characteristics of the system. Complete reactions contained the components described in the text, 15  $\mu\text{mole}$  uridylic acid residues in polyU, and 20.6  $\mu\text{mole}$   $C^{14}$ -Phe-sRNA (2050 count/min, 0.714  $A^{200}$  units). Incubation was at 0°C for 60 minutes. Deacylated sRNA was added either at zero time or after 50 minutes of incubation, as indicated.

Modifications	$C^{14}$ -Phe-sRNA bound to ribosomes ( $\mu\text{mole}$ )
Complete	5.99
- PolyU	0.12
- Ribosomes	0
- $Mg^{++}$	0.09
+ sRNA (deacylated) at 50 min	
0.500 $A^{200}$ units	5.69
2.500 $A^{200}$ units	5.36
+ sRNA (deacylated) at zero time	
0.500 $A^{200}$ units	4.49
2.500 $A^{200}$ units	2.08

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

*Assay of ribosomal bound  $C^{14}$ -aminoacyl-sRNA.* Each 50- $\mu\text{l}$  reaction mixture contained: 0.1M tris-acetate, pH 7.2; 0.02M magnesium acetate; 0.05M KCl; 2.0  $A^{200}$  units of ribosomes (washed three times) and, as indicated for each experiment, oligo- or polynucleotide, and  $C^{14}$ -aminoacyl sRNA. Tubes were kept at 0°C and  $C^{14}$ -aminoacyl sRNA was added last to initiate binding (less binding was obtained if polynucleotide was added last). Incubation for 20 minutes at 24°C was often convenient for studies requiring max-

imum binding, and incubation for 3 minutes at 24°C or 30 minutes at 0°C for rate studies.

After incubation, tubes were placed in ice and each reaction was immediately diluted with 3 ml of buffer containing 0.10M tris-acetate, pH 7.2; 0.02M magnesium acetate; and 0.05M KCl, at 0° to 3°C. A cellulose nitrate filter (HA Millipore filter, 25 mm diameter, 0.45 $\mu$  pore size) in a stainless steel holder was washed under gentle suction with 5 ml of buffer at 0° to 3°C. The diluted reaction mixture was immediately poured on the filter under suction and washed to remove unbound  $C^{14}$ -aminoacyl sRNA with three, 3-ml portions of buffer at 0° 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  $C^{14}$ -counting efficiency of 23 percent. In some experiments, radioactivity was determined in a liquid scintillation counter with a  $C^{14}$ -counting efficiency of 65 percent (21) and dried filters (not glued) were placed in vials containing 10 ml of

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  $\mu\text{l}$ , the specified polynucleotide and  $C^{14}$ -aminoacyl-sRNA (14.7  $\mu\text{mole}$   $C^{14}$ -Phe-sRNA, 2015 count/min, 0.960  $A^{200}$  units; 16.5  $\mu\text{mole}$   $C^{14}$ -Lys-sRNA, 1845 count/min, 0.530  $A^{200}$  units; 30  $\mu\text{mole}$   $C^{14}$ -Pro-sRNA, 2750 count/min, 1.570  $A^{200}$  units).

Polynucleotide ( $\mu\text{mole}$ base residues)	$C^{14}$ -Aminoacyl-sRNA bound to ribosomes ( $\mu\text{mole}$ )		
	$C^{14}$ -Phe-sRNA	$C^{14}$ -Lys-sRNA	$C^{14}$ -Pro-sRNA
None	0.19	0.99	0.25
PolyU, 25	6.00	.67	.15
PolyA, 16	0.22	4.35	.17
PolyC, 19	.21	0.72	.80

toluene-PPO-POPOP phosphor solution (22).

*Preparation of sRNA.* Except where noted *E. coli* B sRNA (23), was used. Uniformly labeled  $C^{14}$ -L-phenylalanine,  $C^{14}$ -L-lysine, and  $C^{14}$ -L-leucine with specific radioactivities of 250, 305, and 160  $\mu\text{C}/\mu\text{mole}$ , 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  $C^{14}$ -amino acyl-sRNA was prepared by modifications of meth-

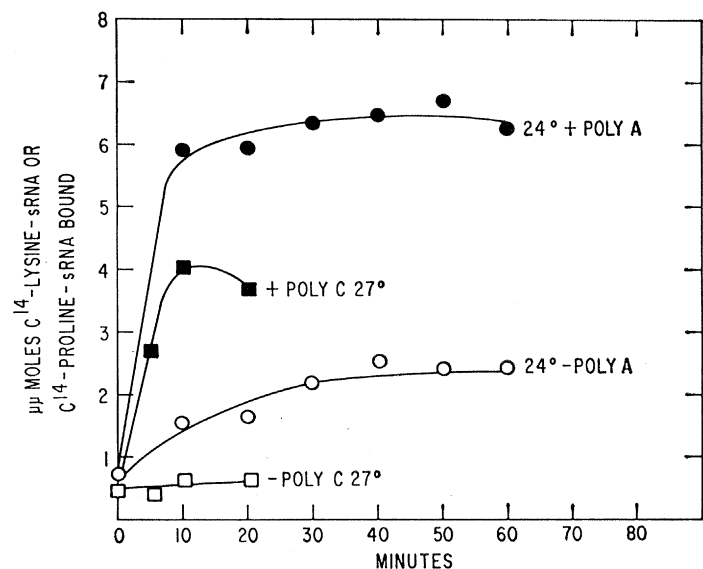
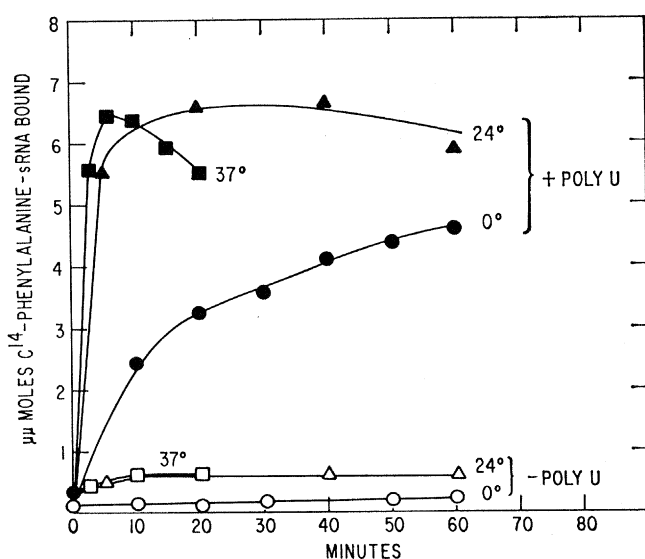


Fig. 1 (left). Effect of polyU upon the rate of  $C^{14}$ -Phe-sRNA binding to ribosomes at 0°, 24°, and 37°C. Each point represents a 50  $\mu\text{l}$  reaction mixture incubated for the time and at the temperature indicated. Reaction mixtures contain the components described under *Methods*; 9.65  $\mu\text{mole}$  of  $C^{14}$ -Phe-sRNA (1180 count/min, 0.380  $A^{200}$ ); and polyU, 25  $\mu\text{mole}$  base residues, where specified. Fig. 2 (right). The effect of polyA and polyC upon the rates of  $C^{14}$ -Lys- and  $C^{14}$ -Pro-sRNA binding to ribosomes, respectively. Each point represents a 50- $\mu\text{l}$  reaction mixture as described under *Methods*. The reactions specified contained 27.5  $\mu\text{mole}$   $C^{14}$ -Lys-sRNA (3080 count/min, 0.880  $A^{200}$  units) and polyA, 25  $\mu\text{mole}$  base residues, or 11.8  $\mu\text{mole}$   $C^{14}$ -Pro-sRNA (2660 count/min, 0.905  $A^{200}$  units) and polyC, 25  $\mu\text{mole}$  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  $C^{14}$ -amino acid plus 19  $C^{12}$ -amino acids. The formation of  $C^{14}$ -aminoacyl sRNA was catalyzed by the supernatant solution obtained by centrifugation of *E. coli* (W-3100) extracts at 100,000g.

**Elution and characterization of  $C^{14}$ -phenylalanine product bound to ribosomes.** Reaction mixtures (0.5 ml) incubated at 24°C for 10 minutes with  $C^{14}$ -Phe-sRNA and polyU were washed on cellulose nitrate filters in the usual manner. The ribosomal bound  $C^{14}$ -product was eluted from filters by washing with 0.01M tris-acetate, pH 7.2;  $10^{-5}$ M magnesium acetate; and 0.05M KCl at 0°C.

The  $C^{14}$ -product eluted from ribosomes was precipitated in 10 percent TCA at 3°C in the presence of 200  $\mu$ g of bovine serum albumin. Specified samples were heated in 10 percent TCA at 90° 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  $NH_4OH$  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  $\mu$ g of purified pancreatic ribonuclease A (purified chromatographically) (27) at 37°C for 15 minutes.

## Results and Discussion

**Assay of ribosomal bound  $C^{14}$ -aminoacyl-sRNA.** The assay is based upon the retention of ribosomes and  $C^{14}$ -aminoacyl sRNA bound to ribosomes by cellulose nitrate filters. After unbound  $C^{14}$ -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  $C^{14}$ -amino acid of specific radioactivity 100 to 300  $\mu$ C/ $\mu$ mole, the binding to ribosomes of 0.2  $\mu$ mole of  $C^{14}$ -aminoacyl sRNA readily can be detected. A filter 25 mm in diameter with a pore size of 0.45  $\mu$  retains up to 1200  $\mu$ g 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  $C^{14}$ -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  $C^{14}$ -Phe-sRNA by the filter. Ribosomes, polyU, and  $Mg^{++}$  were required for retention of  $C^{14}$ -Phe-sRNA. Spyrides (28) and Conway (29) have reported that polyU-directed binding of Phe-sRNA to ribosomes is dependent upon  $K^+$  or  $NH_4^+$ .

The addition of deacylated sRNA to reactions shortly before incubation was terminated, after  $C^{14}$ -Phe-sRNA binding had ceased, had little effect upon ribosomal bound  $C^{14}$ -Phe-sRNA. The bound  $C^{14}$ -Phe-sRNA fraction apparently is not readily exchangeable.

In contrast, the addition of deacylated sRNA at the start of incubation inhibited  $C^{14}$ -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  $C^{14}$ -aminoacyl sRNA (for example, polyA and  $C^{14}$ -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  $C^{14}$ -Phe-,  $C^{14}$ -Lys-, and  $C^{14}$ -Pro-sRNA, respectively. These data agree well with specificity data obtained with a sucrose-gradient 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

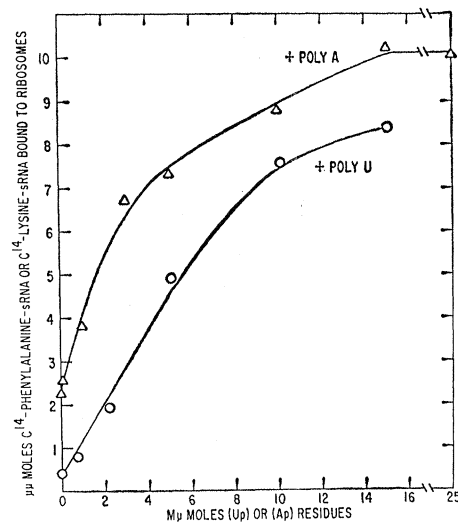


Fig. 3. Relation between polynucleotide concentration and  $C^{14}$ -aminoacyl-sRNA binding to ribosomes. Each point represents a 50- $\mu$ l reaction mixture with the components described under *Methods*;  $\Delta$ , polyA as specified and 27.5  $\mu$ mole  $C^{14}$ -Lys-sRNA (3080 count/min, 0.880  $A^{260}$  units);  $\circ$ , polyU as specified and 15  $\mu$ mole of  $C^{14}$ -Phe-sRNA (2050 count/min, 0.714  $A^{260}$  units). Incubation was at 24°C for 20 minutes.

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  $C^{14}$ -Phe-sRNA binding; how-

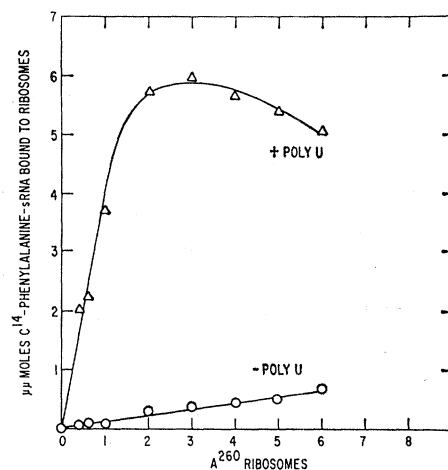


Fig. 4. Relation between ribosome concentration and  $C^{14}$ -Phe-sRNA binding. Each point represents a 50- $\mu$ l reaction mixture containing the amount of ribosomes indicated;  $\circ$  No additions, (-poly U);  $\Delta$  + poly U, 25  $\mu$ mole base residues. In addition, reaction mixtures contain the components described under *Methods* and 9.65  $\mu$ mole of  $C^{14}$ -Phe-sRNA (1180 count/min, 0.380  $A^{260}$ ). Incubations were for 10 minutes at 24°C.

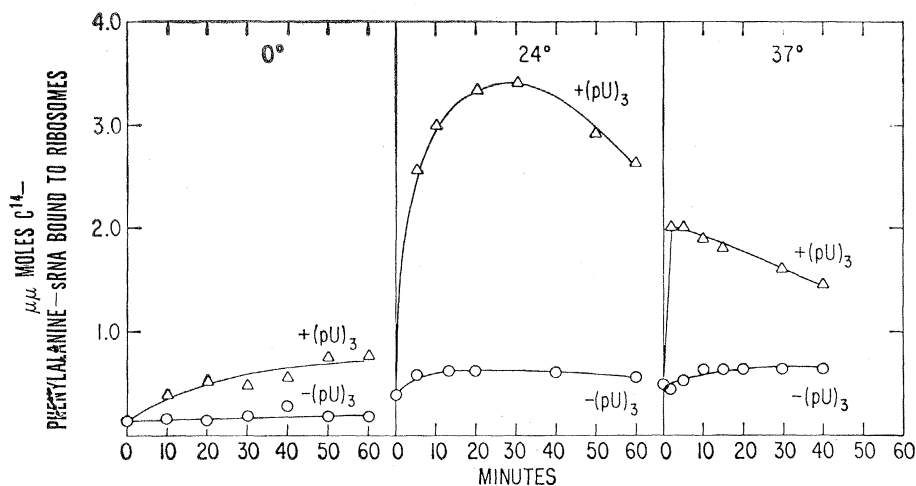


Fig. 5. Effect of pUpUpU upon the rate of  $C^{14}$ -Phe-sRNA binding to ribosomes at  $0^\circ$ ,  $24^\circ$ , and  $37^\circ$ .  $\circ$ , No addition;  $\Delta$ , addition of  $3.67 \mu\text{mole}$  pUpUpU. Each point represents a  $50\text{-}\mu\text{l}$  reaction mixture incubated for the time and at the temperature specified. Reaction mixtures contain the components described under *Methods*;  $9.65 \mu\text{mole}$  of  $C^{14}$ -Phe-sRNA ( $1180 \text{ count/min}$ ,  $0.380 A^{200}$ ); and oligoU as specified.

ever, the rate of binding increased as the temperature of incubation was raised. Although polyU induced  $C^{14}$ -Phe-sRNA binding at  $0^\circ\text{C}$ , maximum binding was not observed after 60 minutes of incubation. Maximum binding concentrations of  $C^{14}$ -Phe-sRNA, 50 minutes of incubation at  $24^\circ\text{C}$  and

after 6 minutes at  $37^\circ\text{C}$ . In this experiment equal amounts of  $C^{14}$ -Phe-sRNA were bound at  $24^\circ$  and  $37^\circ\text{C}$ . In other experiments, with limiting concentrations of  $C^{14}$ -Phe-sRNA, 50 to 98 percent of the  $C^{14}$ -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  $C^{14}$ -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  $C^{14}$ -Lys- and  $C^{14}$ -Pro-sRNA binding to ribosomes is shown in Fig. 2. Maximum stimulation of  $C^{14}$ -Lys-sRNA binding by polyA, and of  $C^{14}$ -Pro-sRNA binding by polyC, occurred after 10 minutes of incubation at  $24^\circ$  and  $27^\circ\text{C}$ , respectively. In this experiment,  $C^{14}$ -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  $C^{14}$ -Phe- or  $C^{14}$ -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  $C^{14}$ -Phe-

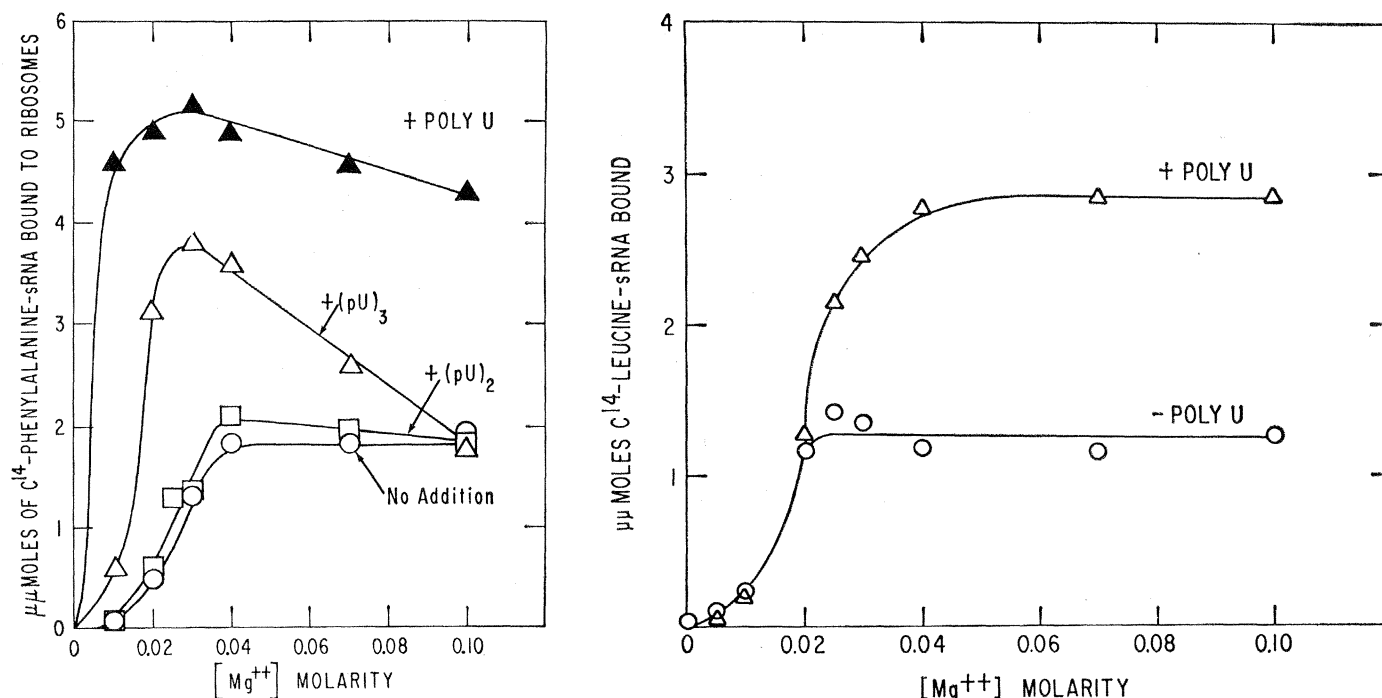


Fig. 6 (left). Effect of  $(\text{Mg}^{++})$  concentration upon  $C^{14}$ -Phe-sRNA binding to ribosomes. Each symbol represents a  $50\text{-}\mu\text{l}$  reaction mixture containing:  $\circ$ , polymer (no addition);  $\square$ , pUpU,  $10 \mu\text{mole}$  base residues;  $\Delta$ , pUpUpU,  $10 \mu\text{mole}$  base residues;  $\blacktriangle$ , polyU,  $25 \mu\text{mole}$  base residues; and the components described under *Methods*; the magnesium acetate concentrations of reaction mixtures and the washing buffer are shown in the figure;  $3.5 A^{200}$  units of ribosomes; and  $8.63 \mu\text{mole}$   $C^{14}$ -Phe-sRNA ( $1025 \text{ count/min}$ ,  $0.357 A^{200}$  units). Incubation was at  $24^\circ\text{C}$  for 20 minutes.

Fig. 7 (right). The polyU-leucine ambiguity. The effect of polyU upon  $C^{14}$ -Leu-sRNA binding to ribosomes at different  $\text{Mg}^{++}$  concentrations. Each  $50\text{-}\mu\text{l}$  reaction contained the components described under *Methods*; the magnesium acetate concentration specified in the figure  $23.7 \mu\text{mole}$   $C^{14}$ -Leu-sRNA ( $1710 \text{ count/min}$ ,  $0.424 A^{200}$  units of *E. coli* W 3100 sRNA, charged only with  $C^{14}$ -leucine); and polyU,  $25 \mu\text{mole}$  base residues where specified. Incubation was at  $24^\circ\text{C}$  for 20 minutes. Each reaction mixture was washed with a buffer containing  $\text{Mg}^{++}$  at the concentration present during incubation.

Table 3. Oligonucleotide specificity. Reaction mixtures containing either  $C^{14}$ -Phe-,  $C^{14}$ -Lys-, or  $C^{14}$ -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.

Oligonucleotide (m $\mu$ mole base residues)	$C^{14}$ -Aminoacyl-sRNA bound to ribosomes ( $\mu\mu$ mole)		
	$C^{14}$ -Phe- sRNA	$C^{14}$ -Lys- sRNA	$C^{14}$ -Pro- sRNA
None	0.34	0.80	0.24
pUpUpU (10)	1.56	0.56	0.20
pApApA (7)	0.20	6.13	0.18
pCpCpC (8)	0.30	0.60	0.73

sRNA binding to ribosomes throughout the pH range tested, from 5.5 to 7.8. Binding was maximum in reactions buffered either with 0.1M tris, pH 7.2, or with 0.05M cacodylate, pH 6.5.

The addition of GTP, PEP, and PEP kinase to reactions, with or without polyU, did not stimulate  $C^{14}$ -Phe-sRNA binding; however, further study is necessary to determine whether binding in this system is dependent upon enzymatic catalysis or GTP or both. Polyphenylalanine synthesis (that is, radioactivity in the fraction precipitable with hot TCA) was not detected in reaction mixtures incubated under optimum conditions for  $C^{14}$ -Phe-sRNA binding in the presence of polyU. In additional experiments,  $C^{14}$ -Phe-sRNA induced by polyU to bind to ribosomes was eluted with  $10^{-5}M$  Mg<sup>++</sup> as already described. Although 50 to 98 percent of the ribosomal-bound  $C^{14}$ -product was eluted (in different experiments), few ribosomes were released from filters. The  $C^{14}$ -product was insoluble in TCA at 3°C, but was converted quantitatively to a soluble product by (i) incubation at pH 10.2 for 60 minutes at 37°C, (ii) heating in TCA, or (iii), incubation with pancreatic ribonuclease. Further characterization of the bound  $C^{14}$ -product is necessary; however, these preliminary results are in accord with the demonstration by Schweet and his co-workers that the binding of Phe-sRNA to ribosomes induced by polyU is not dependent upon peptide-bond formation (6, 7).

The relation between ribosome concentration and the amount of bound  $C^{14}$ -Phe-sRNA is illustrated in Fig. 4.  $C^{14}$ -Phe-sRNA binding was stimulated markedly by polyU and was proportional to the ribosome concentration, within the range 0 to 1.0  $A^{200}$  units of ribosomes. The number of 70S *E. coli*

ribosomes and ribosomal-bound  $C^{14}$ -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  $\mu\mu$ mole of  $C^{14}$ -Phe-sRNA became bound to 23.2  $\mu\mu$ mole of 70S ribosomes (4); therefore, the  $C^{14}$ -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.

*Effect of oligonucleotides on the binding of  $C^{14}$ -aminoacyl sRNA to ribosomes.* OligoU preparations of different chain length were prepared, and their effect upon  $C^{14}$ -Phe-sRNA binding to ribosomes was determined. The effect of the trinucleotide, pUpUpU, upon  $C^{14}$ -Phe-sRNA binding to ribosomes, at 0°, 24°, and 37°C, is shown in Fig. 5. The  $C^{14}$ -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  $C^{14}$ -aminoacyl-sRNA binding to ribosomes and suggest a general method of great simplicity for determining the genetic function of other trinucleotide sequences.

The binding of  $C^{14}$ -Phe-,  $C^{14}$ -Lys-, and  $C^{14}$ -Pro-sRNA was induced with apparent specificity by pUpUpU, pApApA, and pCpCpC, respectively (Table 3). In additional experiments, each trinucleotide had no discernible effect upon the binding to ribosomes of 15 aminoacyl-sRNA preparations, each charged with a different  $C^{14}$ -amino acid ( $C^{14}$ -asparagine and  $C^{14}$ -glutamine-sRNA were not tested). Therefore, the specificity of each trinucleotide for inducing sRNA binding to ribosomes was high and clearly paralleled that of the corresponding polynucleotide.

The  $T_m$  of the interaction between pApApA and polyU is 17°C (33). Therefore, hydrogen-bonding between a triplet codeword in mRNA and a complementary "anticodeword" in sRNA would not by itself appear sufficient to account for the stability of the interaction between  $C^{14}$ -Phe-sRNA, polyU, and ribosomes. An interaction between

Table 4. Template activity of oligodeoxynucleotides. The components of each 50  $\mu$ l reaction mixture are presented in the text. In addition, each reaction mixture in Expt. 1 contained 9.65  $\mu\mu$ mole of  $C^{14}$ -Phe-sRNA (1370 count/min, 0.380  $A^{200}$  units); and in Expt. 2, 16.5  $\mu\mu$ mole  $C^{14}$ -Lys-sRNA (1845 count/min, 0.530  $A^{200}$  units) and the oligonucleotides specified. Mixtures were incubated at 24°C for 10 minutes.

Oligonucleotide (m $\mu$ mole)	$C^{14}$ -Aminoacyl-sRNA bound to ribosomes ( $\mu\mu$ mole)
<i>Experiment 1: <math>C^{14}</math>-Phe-sRNA</i>	
None	0.29
1.00 pUpUpU	1.29
3.33 pUpUpU	2.40
6.67 pUpUpU	2.90
3.33 oligo d(pT) <sub>3</sub>	0.35
6.67 oligo d(pT) <sub>3</sub>	0.39
10.00 oligo d(pT) <sub>3</sub>	0.40
1.67 oligo d(pT) <sub>12</sub>	0.31
2.50 oligo d(pT) <sub>12</sub>	0.44
<i>Experiment 2: <math>C^{14}</math>-Lys-sRNA</i>	
None	1.27
0.17 pApApA	3.48
1.37 pApApA	5.99
3.60 pApApA	7.40
0.65 oligo d(pA) <sub>8</sub>	1.45
1.30 oligo d(pA) <sub>8</sub>	1.68
2.00 oligo d(pA) <sub>8</sub>	1.61

the -CpCpA end of sRNA and ribosomes is possible, for several laboratories (3, 4, 34) have reported that removal of the terminal adenosine of sRNA greatly reduces its ability to bind to ribosomes. The participation of an enzyme in the binding process also must be considered.

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)<sub>3-12</sub> and oligo-d(pA)<sub>8</sub> 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<sup>++</sup> are shown in Fig. 6. Both tri- and polyU induced maximal binding at approximately 0.03M Mg<sup>++</sup> (0.02 to 0.03M in other experiments). Although the dinucleotide, 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<sup>++</sup>, the concentration used throughout our study, little binding of  $C^{14}$ -Phe-sRNA was found in the absence of polyU. However, at higher Mg<sup>++</sup> concentrations, Phe-sRNA binding in the absence of template RNA increased;

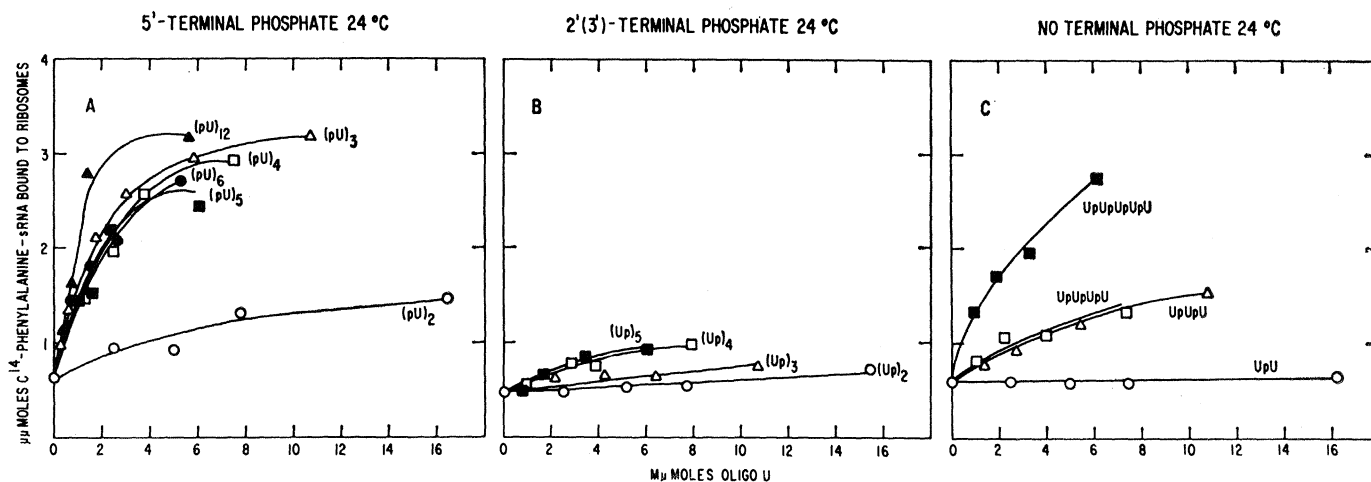


Fig. 8. *A*, *B*, and *C*, Relation between template activity and oligoU chain-length, concentration and end-group. The activities of oligoU with 5'-terminal phosphate are shown in Fig. 8*A*; with 2'(3')-terminal phosphate in Fig. 8*B*; and without terminal phosphate in Fig. 8*C*. Symbols represent oligoU chain-lengths as follows: ○ 2; △ 3; □ 4; ■ 5; ● 6; ▲ 12. Each reaction mixture contained, in a volume of 50  $\mu$ l, the components specified in the *Methods* section; oligoU preparations and concentrations specified; and 10.8  $\mu$ μmole  $C^{14}$ -Phe-sRNA (1880 count/min, 0.714  $A^{260}$ ). Oligonucleotide preparations (pU)<sub>2</sub>, No. 610; (pU)<sub>5</sub>, No. 613; (Up)<sub>1</sub>, No. 617; with the contaminants specified under *Methods* were used in these experiments. Incubations were at 24°C for 30 minutes.

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  $C^{14}$ -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  $C^{14}$ -Leu-sRNA. It is possible that the  $Mg^{++}$ -dependent leucine-polyU ambiguity occurs before peptide-bond synthesis.

As shown in Table 5,  $C^{14}$ -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, pUpUpU had no effect upon the binding of  $C^{14}$ -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. 8*A*; preparations with 2'(3')-terminal phosphate in Fig. 8*B*; and preparations without terminal phosphate, in Fig. 8*C*.

As shown in Fig. 8*A*, the dinucleotide with 5'-terminal phosphate, pUpU, had little template activity, whereas the trinucleotide, pUpUpU, markedly stimulated  $C^{14}$ -Phe-sRNA binding. This ob-

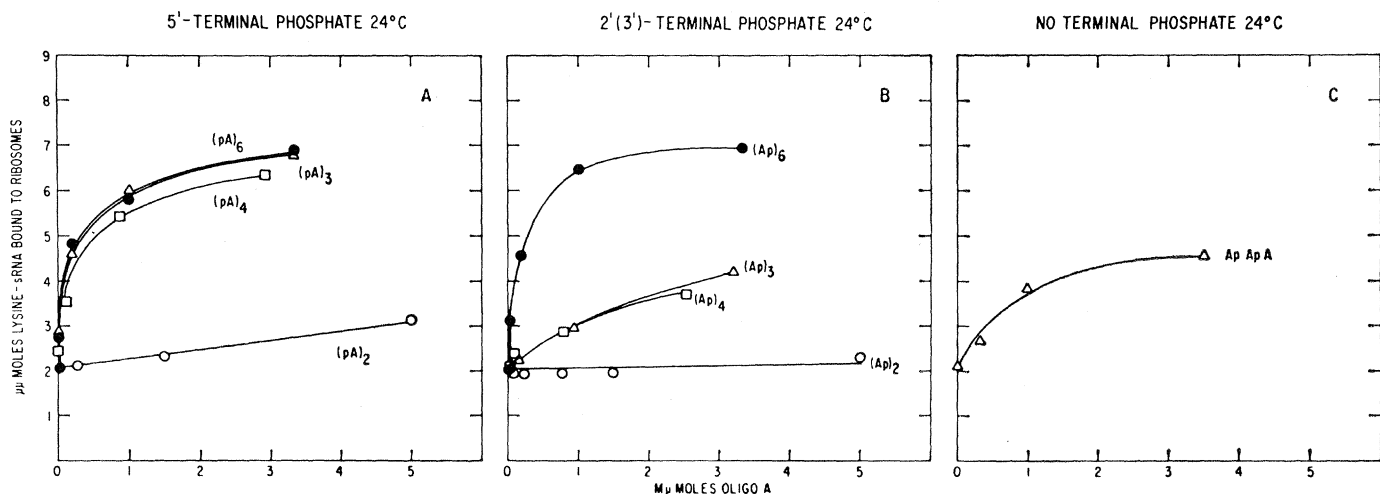


Fig. 9. *A*, *B*, and *C*, Relation between template activity and oligoA concentration, chain-length, and end group. The activity of oligoA with 5'-terminal phosphate is shown in Fig. 9*A*; preparation without terminal phosphate in Fig. 9*C*. The symbols indicate the chain-length of oligoA as follows: ○ 2; △ 3; □ 4; ● 6. Each 50  $\mu$ l reaction mixture contained the components specified under methods; 15.9  $\mu$ μmole of  $C^{14}$ -Lys-sRNA (1780 count/min, 0.562  $A^{260}$  units); and the oligoA preparation specified. Incubation was at 24°C for 20 minutes. Oligonucleotide preparations (pA)<sub>6</sub>, No. 599 and (Ap)<sub>4</sub>, No. 591, containing the contaminants specified under *Methods*, were used in this experiment.

servation provides direct experimental support for a triplet code for phenylalanine 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<sup>14</sup>-Phe-sRNA to ribosomes (Fig. 8B). The template activity of the tetra- and pentanucleotide 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<sup>14</sup>-Phe-sRNA binding, but less actively than pUpUpU. The template activity of the pentamer, UpUpUpUpU, was almost equal to that of pUpUpUpU.

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<sup>14</sup>-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<sup>14</sup>-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 in-

active as a template for C<sup>14</sup>-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'- towards 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<sup>14</sup>-Val-sRNA binding to ribosomes, whereas UpUpG did not.

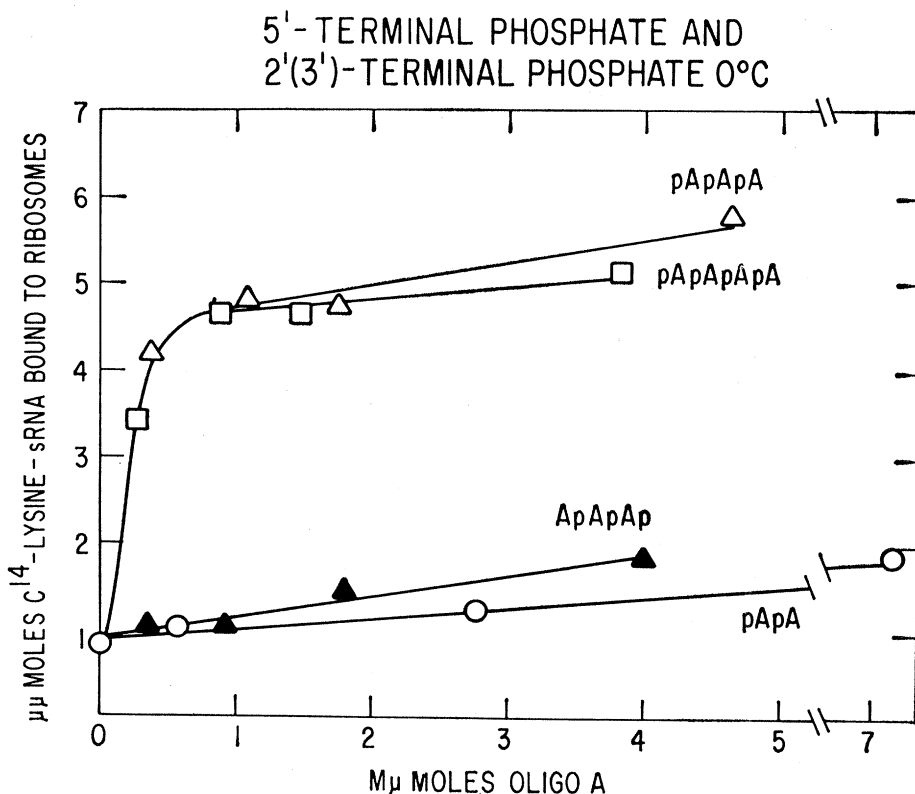


Fig. 10. Template activity of oligoA with 5'- or 2'(3')-terminal phosphate in reaction mixtures incubated at 0°C for 60 minutes. OligoA concentrations, chain-lengths, and end-groups are shown in the figure. Each 50-μl reaction mixture contained the components specified under *Methods*; 11.8 μμmole C<sup>14</sup>-Lys-sRNA (2840 count/min, 0.767 A<sup>290</sup> units); and the oligoA preparation specified.



## Regulatory Codewords

**Terminal codewords.** We suggest that 5'-terminal, 3'-terminal and internal codewords of RNA and DNA constitute separate classes of codewords, for each differs in chemical structure as shown diagrammatically in Fig. 11. The 5'-terminal codeword contains a 5'-terminal hydroxyl, the internal codeword is attached to adjacent codewords on both sides by way of (3'-5')-phosphodiester bonds, and the 3'-terminal codeword contains 2'- and 3'-terminal hydroxyl groups. It should be noted that phosphate, linked to a terminal hydroxyl is a monoester, whereas, an internal phosphate is a diester. Therefore, the 5'-terminal, 3'-terminal, and internal codewords differ in chemical structure. To avoid confusion between the three codeword classes, the codeword with free or substituted 5'-hydroxyl will be designated the 5'-terminal codeword, and the codeword with a free or substituted 3'-hydroxyl, will be designated the 3'-terminal codeword. Internal codewords will not be designated by position. These differences raise the possibility that codewords may occur in three chemically distinct forms and that RNA and DNA may contain either one, two, or three forms of any triplet codeword, depending upon the position of the codeword in the molecule.

### *Sense-missense-nonsense codewords.*

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

Table 5. Specificity of polyU-leucine-sRNA bound to ribosomes. Reaction mixtures contained 0.07M magnesium acetate; other components as described in the text; 3.1  $\mu\mu\text{moles}$  of  $\text{C}^{14}$ -Leu-sRNA (2280 count/min, 0.565  $A^{260}$  units of *E. coli* W 3100 sRNA acylated only with  $\text{C}^{14}$ -Leucine); and oligo- or polynucleotides as specified. Incubations were at 24°C for 20 minutes. The magnesium acetate concentration of the solution used to wash ribosomes on filters after incubation was 0.07M.

Addition (base residues) ( $\mu\mu\text{mole}$ )	$\text{C}^{14}$ -Leu-sRNA bound to ribosomes ( $\mu\mu\text{mole}$ )
None	1.00
25 PolyU	2.02
15 pUpUpU	0.70
25 PolyA	.99
25 PolyC	.85

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.

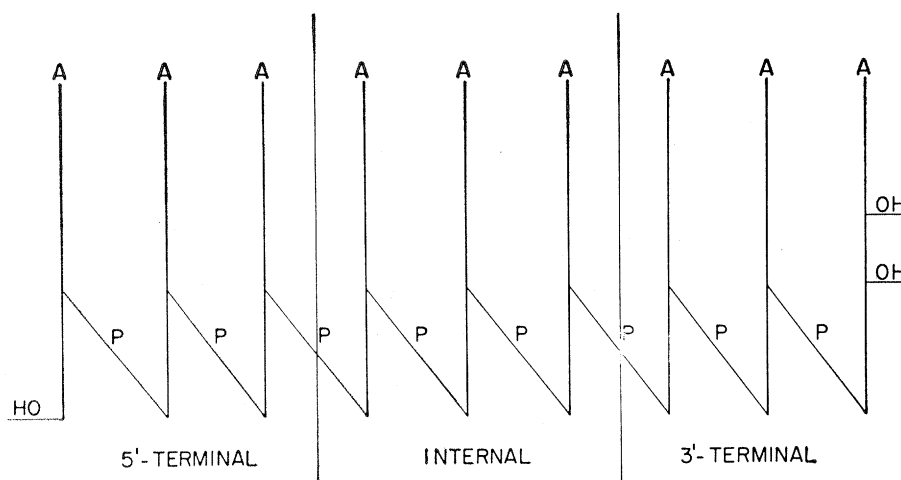


Fig. 11. The structures of three classes of codewords, 5'-terminal, internal, and 3'-terminal, are illustrated diagrammatically. The figure represents oligoA of chain-length 9, ApApApApApApApApA. Although the three triplet codewords have identical base-sequences, AAA, each differs in structure. The 5'-terminal codeword contains a 5'-terminal hydroxyl; the internal codeword is linked to adjacent codewords on either side by way of 3', 5'-phosphodiester bonds; and the 3'-terminal codeword contains 2', 3'-terminal hydroxyl groups (3'-hydroxyl only if deoxyribose). Phosphate, when present at 5'-, 3'-, or 2'-terminal hydroxyl positions, may be a phosphomonoester, and therefore may differ from an internal phosphate which is a phosphodiester.

## Summary

A rapid, sensitive method is described for measuring  $C^{14}$ -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, pApApA, and pCpCpC, 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 trinucleotide codewords is modified greatly by terminal phosphate. Triplets with 5'-terminal phosphate are more active as templates than triplets without terminal phosphate. Triplets with 3'- or 3' (2')-terminal phosphate are markedly less active as templates. These findings are discussed in relation to the probable functions of terminal codewords. The modification of RNA and DNA codewords, converting sense into 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, polyadenylic acid; TCA, trichloroacetic acid; d(pT), deoxythymidylic acid; d(pA), deoxyadenylic acid; sRNA, transfer RNA;

- mRNA, messenger RNA; DEAE, diethylaminoethyl cellulose; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5'-phenyloxazolyl) benzene; PEP, phosphoenolpyruvate;  $T_m$ , melting temperature;  $A^{260}$ , at 260 m $\mu$ .
- For mono- and oligonucleotides of specific structure, the p to the left of a terminal nucleoside initial indicates a 5'-terminal phosphate; the p to the right, a 2' (3')-terminal phosphate. Internal phosphates of oligonucleotides are (3'-5')-linkages.
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