# STIMULATION OF AMINO ACID INCORPORATION INTO PROTEIN BY NATURAL AND SYNTHETIC POLYRIBONUCLEOTIDES IN A MAMMALIAN CELL-FREE SYSTEM

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Nirenberg and Matthaei[1-3] have recently described a cell-free system from *E. coli* in which the incorporation of amino acids into protein is dependent on the addition of natural or synthetic template RNA. Our group,[4, 5] as well as Dr. Ochoa's[6-8] has utilized synthetic polyribonucleotides with varying base compositions in this, or a similar system, to investigate the RNA coding units for amino acids. Thus, codes of unspecified base sequence have been determined for 19 of the 20 naturally occurring amino acids.

Comparison of the experimentally determined coding units in *E. coli* with amino acid replacement data in tobacco mosaic virus[4, 8] suggests that the code may apply to species other than *E. coli*. Such generality is implied also by the finding of von Ehrenstein and Lipmann[9] that hemoglobin can be synthesized from amino-acyl-sRNA derived from *E. coli* in a system containing ribosomes from rabbit reticulocytes. Furthermore, Tsugita *et al.*[10] have reported that a system containing *E. coli* ribosomes is capable of synthesizing a protein very similar to that in tobacco mosaic virus provided it is supplied with tobacco mosaic virus-RNA. Recently Arnstein *et al.*[11] have reported a specific stimulation of the incorporation of phenylalanine into protein by polyuridylic acid in a cell-free system from rabbit reticulocytes. Weinstein and Schechter[12] have obtained similar results with several mammalian systems. The latter workers have also utilized synthetic polymers containing more than one base.

The present communication presents additional results with a mammalian cell-free system in which the incorporation of amino acids into protein is stimulated by naturally occurring or synthetic polyribonucleotides. Microsomal RNA (mRNA) stimulates the incorporation of all the amino acids thus far tested. Polyuridylic acid (poly U) specifically stimulates the incorporation of phenylalanine. Polyuridylic-guanylic acid (poly UG) stimulates phenylalanine, leucine, valine, glycine, and tryptophan, and polyuridylic-cytidylic acid (poly UC) stimulates phenylalanine, leucine, and serine. The data constitute direct evidence that at least a part of the code previously determined for *E. coli* applies as well to a completely mammalian system and lend additional support to the existence of a universal code.

*Materials and Methods.*—The system employed was essentially that described by Keller and Zamecnik[13] except that medium A and medium B were modified to contain 0.07 *M* KCl and $6 \times 10^{-3}$ *M* mercaptoethanol. Liver microsomes and pH 5 enzymes were prepared fresh daily from four male Sprague-Dawley rats weighing approximately 100 gm each. Incubation mixtures routinely contained 0.35 *M* sucrose, 0.07 *M* KCl, 0.05 *M* Tris buffer pH 7.8, 4–6 $\times 10^{-3}$ *M* $MgCl_2$, $1 \times 10^{-3}$ *M* ATP, $6 \times 10^{-3}$ *M* mercaptoethanol, $1 \times 10^{-4}$ *M* GTP, $5 \times 10^{-3}$ *M* potassium phosphoenolpyruvate, 20 $\mu$g of crystalline phosphoenolpyruvate kinase (Sigma Chemical Co.), $8 \times 10^{-5}$ *M* $C^{14}$-amino acid, $1 \times 10^{-4}$ *M* each of 19 L-amino acids minus the $C^{14}$-amino acid, 1.5 mg of ribosomal protein, 2.5 mg of pH 5 fraction protein, and mRNA, sRNA, or synthetic polyribonucleotides as indicated. The final volume was 0.5 ml and incubation was at 37° for 30 min. Protein was precipitated by the addition of 0.5 ml of 10% TCA and the precipitate

was washed according to the method of Siekevitz.[14] The precipitate was dissolved in 1 ml of a 1 *M* methanolic solution of hydroxide of hyamin (Packard Instrument Co.), added to 10 ml of 0.6% 2,5-diphenyloxazole in toluene, and counted in a Packard liquid scintillation counter. Under the specified conditions, one $\mu$c gave $1.46 \times 10^6$ cpm, a counting efficiency of approximately 65%. All assays were performed in duplicate.

U-$C^{14}$ L-amino acids and $C^{14}$ DL-tryptophane were obtained from Nuclear-Chicago Corporation. The specific activities are given in Table 3. Poly U was obtained from Miles Chemical Laboratories. Poly UG and poly UC with known base ratios synthesized enzymatically with polynucleotide phosphorylase from *Micrococcus Lysodeckticus*,[15] were the generous gifts of Marshall Nirenberg and O. W. Jones. Rat liver mRNA and sRNA were prepared by the phenol extraction procedure of Kirby[16] as described by Hoagland *et al.*[17] Ribonuclease was obtained from Worthington Biochemical Corporation.

*Results and Discussion.*—Figure 1*A* shows the effects of increasing concentrations

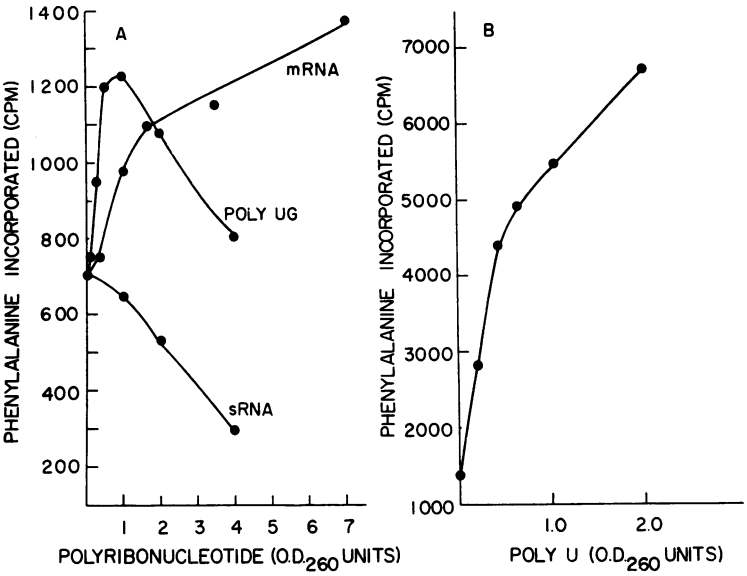


FIG. 1.—The effects of increasing concentration of mRNA, sRNA, poly UG, and poly U on the incorporation of phenylalanine into protein. The reaction mixtures are described in *Methods*.

of mRNA, sRNA, or poly UG on the incorporation of phenylalanine into protein. Both mRNA and poly UG stimulated the reaction, presumably by serving as templates. The relatively large amount of mRNA required for stimulation is consistent with the concept that only a small fraction of the total microsomal RNA can function as template. sRNA inhibited the incorporation of phenylalanine. A similar inhibition by sRNA of the incorporation of leucine into protein in a system from rat liver was recently reported by Decken and Campbell.[18] In the present experiment sRNA not only inhibited the system but also abolished the stimulation by poly UG. No stimulation by 0.5 $OD_{260}$ unit of poly UG was obtained in the presence of 2.0 $OD_{260}$ units of sRNA. The reason for these results is not yet known.

The effect of increasing concentrations of poly U is shown in Figure 1*B*. The incorporation of phenylalanine in the absence of added polyribonucleotide was higher in this experiment than in that shown in Figure 1*A*. Such variations were observed with different preparations of microsomes and pH 5 enzymes even though conditions were maintained as nearly constant as practical. Direct comparisons

could be made only between determinations made at the same time with the same preparations of enzymes. Variations were also observed with different preparations of poly U. Different batches obtained from the same source varied widely in their ability to stimulate phenylalanine incorporation. Similar variations have been observed with the system from *E. coli*[4] and can be explained at least in part by variations in the average molecular weights of the polymers.

The results of an attempt to reduce the amino acid incorporation in the absence of added template RNA by preincubation in the absence of $C^{14}$-amino acid are shown in Table 1. Preincubation inactivated the system but only slight reactivation was obtained by the addition of mRNA.

TABLE 1

EFFECT OF PREINCUBATION ON INCORPORATION OF LEUCINE

| Preincubation without $C^{14}$ leucine | Microsoma RNA added | $C^{14}$ leucine incorporated (cpm) |
|---|---|---|
| None | None | 3,120 |
| " | 7.4 $OD_{260}$ units | 4,285 |
| 15 min 37° | None | 165 |
| " | 7.4 $OD_{260}$ units | 300 |

The incubation mixture is given in *Methods*.

The polyribonucleotide-stimulated incorporation of amino acid into protein is inhibited by puromycin and by ribonuclease (Table 2).

TABLE 2

INHIBITION OF POLY UG-STIMULATED INCORPORATION OF PHENYLALANINE BY PUROMYCIN AND RNAASE

| Inhibitor added | Phenylalanine Incorporated (cpm) Without polynucleotide | Poly UG (3:1) | Increase with UG (cpm) |
|---|---|---|---|
| None | 1,145 | 1,740 | 595 |
| Puromycin (2.5 μg) | 60 | 210 | 150 |
| RNAase (10 μg) | 0 | 10 | 10 |

The incubation mixture is described in *Methods*. Where indicated 0.37 $OD_{260}$ unit of poly UG was added.

The amino acid specificity of polyribonucleotide-stimulated incorporation into protein is shown in Table 3. Small but significant stimulations of the incorporation of all the amino acids tested were obtained with mRNA. Poly U specifically stimulated the incorporation of phenylalanine. Poly UG stimulated the incorporation of phenylalanine, leucine, valine, glycine, and tryptophan while poly UC stimulated phenylalanine, leucine, and serine. These results are qualitatively the same as those obtained with the system from *E. coli*[4-8] with the exception that no stimulation by poly UC of proline incorporation has yet been observed in the system from rat liver.

The quantitative aspects of determining the composition of the RNA coding units in the mammalian system are less favorable than in the bacterial system for the following reasons: (1) the observed stimulations by synthetic polyribonucleotides thus far have been smaller, (2) it has not yet been possible to reduce the incorporation in the absence of added template RNA without inactivating the system, (3) rat liver microsomes are inactivated by dialysis and trace amounts of free amino acids cannot be removed. In connection with the last difficulty, the following

TABLE 3

AMINO ACID SPECIFICITY OF POLYRIBONUCLEOTIDE STIMULATED INCORPORATION INTO PROTEIN

| Amino acid | Specific activity mc/mmole | $C^{14}$-Amino Acid Incorporated ($\mu\mu$mole) | | | | |
|---|---|---|---|---|---|---|
| | | Without polynucleotide | Increase stimulated by | | | |
| | | | U | UG (1.6:1)* | UC (3:1)* | mRNA |
| Phenylalanine | 10.0 | 60 | 170 | 150 | 135 | 35 |
| Leucine | 7.7 | 250 | <10 | 110 | 60 | 90 |
| Valine | 6.5 | 95 | <10 | 140 | <10 | 40 |
| Glycine | 5.0 | 135 | <10 | 50 | — | 50 |
| Tryptophane | 8.0 | 30 | <10 | 50 | — | 10 |
| Serine | 2.9 | 170 | <10 | <10 | 60 | 60 |
| Proline | 5.7 | 130 | <10 | <10 | <10 | 35 |
| Lysine | 5.0 | 200 | <10 | <10 | — | 50 |
| Tyrosine | 5.0 | 80 | <10 | <10 | — | 30 |
| Alanine | 4.2 | 230 | <10 | — | — | 20 |
| Threonine | 6.0 | 100 | <10 | — | <10 | 50 |
| Isoleucine | 6.2 | 85 | <10 | <10 | — | — |

Reaction mixture is described in *Methods*. The quantities of poly U, poly UG, poly UC, and mRNA were 0.5, 0.5, 1.3, and 5.0 $OD_{260}$ units respectively. The values recorded for incorporation in the absence of polyribonucleotides were obtained with the same enzyme preparations used for those with mRNA and have been subtracted to obtain the value recorded for stimulation by mRNA. The values recorded for stimulation by poly U, UG, and UC were obtained with different preparations and the appropriate control values have been subtracted in each case.

* Base ratio.

— indicates experiment not performed.

amino acids were tested at $1.6 \times 10^{-4}M$ as well as with the concentration routinely used ($8 \times 10^{-5}M$) and no increase in incorporation of radioactivity was observed: phenylalanine, leucine, valine, glycine, proline, lysine, and threonine. These results indicated that some quantitation might be possible. This is borne out by the results of an experiment with UG copolymers containing varying base ratios. The theoretical frequency of each triplet relative to 3U was calculated assuming random distribution of bases as described by Matthaei *et al.*[4] and is recorded in Table 4. The incorporation of leucine, valine, tryptophan, and glycine stimulated by the

TABLE 4

AMINO ACID INCORPORATION INTO PROTEIN STIMULATED BY UG COPOLYMERS WITH VARYING BASE RATIOS

| Base ratio U:G | Phenylalanine ($\mu\mu$moles) | Amino Acid Incorporated % Relative to Phenylalanine (100%) | | | | Probability of triplet relative to 3U | |
|---|---|---|---|---|---|---|---|
| | | Leucine | Valine | Tryptophane | Glycine | 2U1G | 1U2G |
| 1.6:1 | 150 | 72 | 92 | 33 | 33 | 64 | 41 |
| 6.7:1 | 360 | 20 | 22 | 4 | 6 | 15 | 2.3 |
| 8.2:1 | 375 | 9 | 8 | 2 | — | 12 | 1.5 |
| 10.7:1 | 400 | 4 | 3 | — | — | 9.4 | <1 |
| 26:1 | 380 | 0 | 0 | — | — | 3.9 | <1 |

The reaction mixtures are described in *Methods*. The quantity of UG (1.6:1) added was 0.5 $OD_{260}$ unit. The quantity of the other UG polymers used was 1.0 $OD_{260}$ unit.

various polymers is given as per cent relative to the stimulation of phenylalanine incorporation by the same polymer. Comparison of the stimulation of the various amino acids relative to phenylalanine with the theoretical frequency of triplets containing 2U1G or 1U2G relative to 3U indicates that the coding units for leucine and valine contain 2U1G and those for tryptophane and glycine contain 1U2G. This is in complete agreement with the results obtained with a bacterial system.[4-8] Similar calculations based on the results shown in Table 3 with a single UC copolymer indicate that triplets containing 2U1C also can code for leucine and for serine as well. Thus, assuming triplet codes, the results for at least 6 amino acids (phenyl-

alanine, 3U; leucine, 2U1G or 2U1C; valine, 2U1G; glycine 1U2G; tryptophan 1U2G; and serine 2U1C) are the same in a mammalian and a bacterial system.

In both species, coding units containing either U and G or U and C direct the incorporation of leucine into protein. The fact that this same degeneracy is observed seems of particular interest.

Although some variations may be observed in different species as investigations are extended, the present data strongly suggest that at least a part of the code is universal.

*Summary.*—A mammalian cell-free system has been described in which the incorporation of $C^{14}$-amino acids into protein is stimulated by microsomal RNA and by synthetic polyribonucleotides of known base ratios. The RNA coding units corresponding to six amino acids were determined. These are in complete agreement with results reported for the composition of the coding units for the same amino acids in a system from *E. coli*. The code for leucine is degenerate in both species. The results strongly suggest that at least a part of the code is universal.

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