*MISCODING IN A CELL-FREE SYSTEM FROM SPLEEN**

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Communicated by George Wald, June 26, 1968

Infidelity of translation of the genetic code has often been widely reported[1-12] in cell-free, protein-synthesizing systems derived from bacteria and, much less extensively,[13-17] in cell-free systems. However, the degree by which experimental conditions *per se* contribute to the observed level of infidelity remains undetermined. Nevertheless, some studies on the influence of streptomycin on protein biosynthesis show that this drug causes miscoding only in cell-free systems which contain ribosomes derived from streptomycin-sensitive cells.[9, 11, 18-21] These findings suggest that the phenomenon observed in cell-free systems reflects the *in vivo* process.[12, 21] Thus, it seems likely that the streptomycin case exemplifies a means whereby a specific, externally administered factor can influence the fidelity of *in vivo* protein biosynthesis.

This paper examines the miscoding properties of cell-free systems derived from rabbit spleen, a tissue capable of antibody production. Investigation of such a system is of special interest since the small differences in amino acid composition among antibodies which have been observed,[22-26] and which may be the basis of antibody specificity, could result from miscoding.

The experiments described here were conducted with a ribosomal preparation which had been preincubated in order to eliminate endogenous incorporation (incorporation due to residual native messages), thus avoiding possible ambiguities in interpreting the role of the added synthetic polynucleotide. This treatment is important for studying miscoding; however, no mammalian system previously described has been completely freed of endogenous incorporation without the removal of most of its activity.

Materials and Methods.—Preparation of ribosomes: Rabbit spleens were washed in a solution containing 0.07 *M* KCl, 0.0055 *M* $MgCl_2$, 0.05 *M* tris(hydroxymethyl)aminomethane (Tris) buffer pH 7.6, 0.35 *M* sucrose, 0.006 *M* β-mercaptoethanol (medium A), then weighed, sliced into small pieces, and suspended in medium A 1:2.5 (w/v). The suspension was homogenized carefully in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 18,000 × *g* for 10 min. To the upper two thirds of the supernatant fraction Na-DOC (sodium deoxycholate) was added to give a concentration of 1%, then diluted 1:1 (v/v) in a solution equivalent to medium A but containing 0.9 *M* sucrose instead of the usual level (medium B) and centrifuged at 150,000 × *g* for 90 min. The ribosomal pellet was suspended in medium A and stored at −80°C.

Preparation of pH 5 fraction: Rat livers or rabbit spleens were subjected to the procedure described above, except that no Na-DOC was added. The upper two thirds of the last supernatant was collected and the pH brought to 5.1 by addition of 1 *N* acetic acid. After 30 min of continuous stirring, the precipitate was centrifuged out (10 min at 18,000 × *g*), dissolved in medium A, and stored at −80°C. (The use of rat liver pH 5 fraction is just a matter of convenience; preliminary experiments showed that pH 5 fraction prepared from rat liver is interchangeable with the same fraction from spleen.)

All the operations were carried out at 4°C or below.

Incubation conditions: The reaction mixture in a final volume of 0.1 ml contained in millimoles: KCl, 8; Tris buffer, 5 (pH 7.6); sucrose, 7; β-mercaptoethanol, 0.1; adenosine 5′-triphosphate, 0.16; guanosine 5′-triphosphate, 0.04; and phosphoenolpyruvate,

0.44. It also contained 50 μg of rabbit spleen ribosomal protein, 100 μg of rat liver pH 5 fraction protein, 2 μg of pyruvate kinase, and either 0.88 mμmole of C^{14}-L-phenylalanine, or 0.2 mμmole of C^{14}-L-leucine plus 0.6 mμmole of cold L-phenylalanine, or 50 μg C^{14}-L-leucyl transfer RNA (tRNA) (130 $\mu\mu$moles C^{14}-L-leucine/1 mg tRNA) plus 0.6 mμmole of cold L-phenylalanine. Concentrations of $MgCl_2$, poly U, and poly UC are specified in the figure legends. This mixture was incubated at 35°C for 30 min.

Preincubation conditions: The reaction mixture was the same as for the incubation but contained a complete mixture of cold amino acids (0.075 mM of each) substituting for the labeled amino acids with a $MgCl_2$ concentration of 0.007 *M*. Preincubation was done at 35°C for $1^1/_2$ hr and the mixture was then diluted 1:2 (v/v) in medium B, spun down, suspended, and stored as described above.

Assay of radioactivity: The incorporation was stopped by addition of 3 ml of 5% trichloroacetic acid (TCA) containing an excess of unlabeled L-leucine and L-phenylalanine, and 0.15 mg of protein was added as a carrier. The precipitates were centrifuged, resuspended in 3 ml of 5% TCA, and held in a water bath at 85°C for 30 min. After they were cooled, the samples were recentrifuged, resuspended in 5% TCA, and finally collected on Millipore filters. The filters were washed with 25 ml of 5% TCA, dried, and counted in a Nuclear-Chicago low-background gas-flow counter. Protein concentration was determined by the method of Lowry *et al.*, with bovine serum albumin as a standard.[27]

Chemicals: Radiochemicals and biochemicals used in these experiments were from the following sources: C^{14}-L-phenylalanine, 28.2 mc/mmole, from Radiochemical Centre, Amersham, England; C^{14}-L-leucine, 222 mc/mmole, from New England Nuclear Corp.; poly U (polyuridylic acid-potassium salt), from Miles Chemical Corp. Poly-UC (1:1 input) was kindly donated by Dr. Y. Kimhi.[28] C^{14}-L-leucyl-tRNA was prepared according to the procedure of V. Daniel.[29]

Results.—Although poly U, a template for polymerization of polyphenylalanine, is not expected to code for leucine, addition of this synthetic polynucleotide to subcellular bacterial systems sometimes stimulates leucine incorporation.[1, 2, 4, 6] As will be shown, the same sort of miscoding property is observed in a subcellular rabbit spleen system.

Figure 1 shows the extent of both phenylalanine and leucine incorporation in the presence and absence of either poly U or poly UC at various Mg^{++} concentrations. As can be seen, addition of poly U to an incubation mixture does stimulate leucine incorporation, but at a much lower level than poly UC does. The magnitude of the incorporation is, however, highly dependent on Mg^{++} concentration. When poly U serves as template, the optimal Mg^{++} concentration for phenylalanine incorporation is obtained at 0.015 *M*, whereas for leucine an optimum of 0.02 *M* is observed. The dependence of leucine incorporation on Mg^{++} concentration with poly UC as template is entirely different. Here, leucine incorporation increases sharply (with the Mg^{++} up to 0.001 *M*) and then declines. Thus at 0.001 *M* Mg^{++}, where poly UC-directed leucine incorporation is at or near optimum, we detect no poly U-dependent leucine uptake. A stimulating effect by poly UC on leucine incorporation is expected, because CUU is one of the codons for leucine,[30, 31] whereas the effect of poly U is not expected and is regarded as miscoding. It should also be noted that the poly U-directed leucine uptake into the polypeptide is very small, relative to its effect on phenylalanine incorporation. As is also shown in Figure 1 (*lower curves*), endogenous incorporation is almost completely abolished by preincubation of the ribosomes (see *Materials and Methods*).

The amount of poly U-directed leucine incorporation (miscoding) is relatively

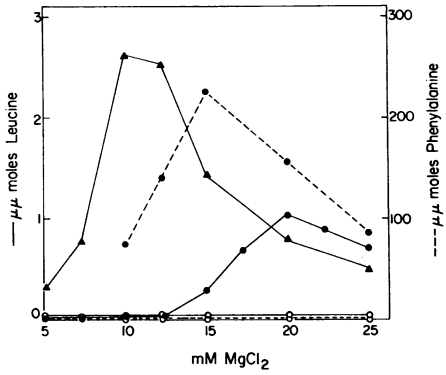

FIG. 1.—Effect of magnesium concentration on the incorporation of C^{14}-leucine and C^{14}-phenylalanine in the presence and absence of poly U or poly UC with preincubated ribosomes. *Continuous lines*, C^{14}-leucine incorporation. *Dashed lines*, C^{14}-phenylalanine incorporation. *Open circles*, endogenous incorporation. *Closed circles*, + 15 μg poly U. *Triangles*, + 15 μg poly UC.

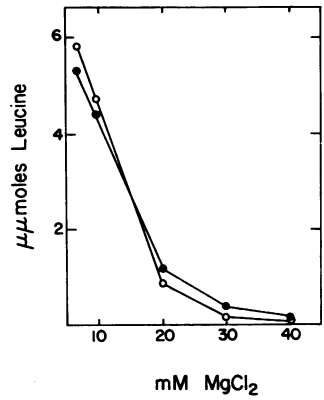

FIG. 2.—Effect of magnesium concentration on incorporation of C^{14}-leucine in the absence and presence of poly U, with the use of untreated ribosomes. *Open circles*, endogenous incorporation. *Closed circles*, + 15 μg poly U.

small in comparison with the endogenous incorporation. In fact, when untreated ribosomes were used, the miscoding could hardly be detected. As is evident from Figure 2, endogenous incorporation masks any poly U-dependent leucine incorporation. Moreover, in the peak region of the endogenous incorporation, addition of poly U seems actually to inhibit leucine incorporation. These results emphasize the need to abolish endogenous incorporation prior to testing for miscoding.

That incorporation of leucine is dependent on poly U concentration is shown in Figure 3. Apparently, the miscoding phenomenon is observed over all poly U concentrations. In addition, incorporation is typically dependent on the amount of ribosomes present. At an input of 15 μg of poly U, 50 μg of ribosomal protein per reaction mixture was found to be optimal (Fig. 4).

In order to avoid any possibility that the magnesium ion effect on miscoding was due to interference with tRNA charging, an experiment in which C^{14}-leucyl-tRNA was substituted for C^{14}-leucine was carried out. As can be seen in Figure 5, the results are qualitatively similar to those depicted in Figure 1. This outcome excludes the possibility that the effect of Mg^{++} on poly U-dependent incorporation is at the level of tRNA charging.

All attempts to detect miscoding involving amino acids other than leucine, or leucine under different conditions, failed. One such negative trial will be summarized. Ethanol and other organic solvents are known to enhance miscoding in bacterial subcellular systems.[1-3] Such effects have been found to be temperature-sensitive.[4, 5] However, ethanol does not react this way in the rabbit spleen system. The pattern of incorporation of either leucine (Fig. 6*A*) or phenylalanine (Fig. 6*B*) by untreated ribosomes in the presence or absence of

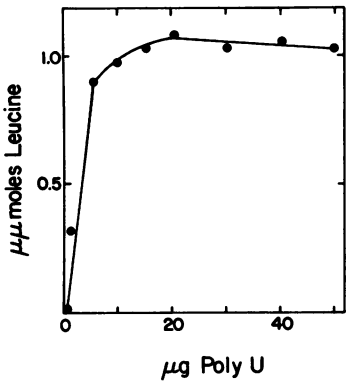

FIG. 3.—Effect of poly U concentration on C^{14}-leucine incorporation. The ribosomes were preincubated. $MgCl_2$ concentration in the incubation mixture was 0.02 *M*.

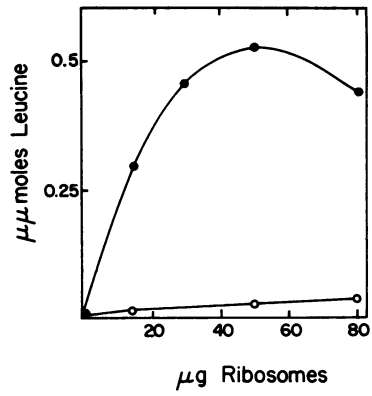

FIG. 4.—Effect of ribosome concentration on C^{14}-leucine incorporation in the absence and presence of poly U. The ribosomes were preincubated. $MgCl_2$ concentration in the incubation mixture was 0.02 *M*. *Open circles*, endogenous incorporation. *Closed circles*, + 15 μg poly U.

poly U is entirely different from the pattern obtained with the bacterial system.[1, 3] The same ethanol concentration that promotes incorporation in bacterial extracts is inhibitory to this mammalian subcellular system. This is true both at 25°C and at 35°C. As shown in Figure 6*A*, ethanol does not cause leucine miscoding with preincubated ribosomes. In the same way, when other materials, such as acetone, dioxane, urea, and streptomycin, were tested for their effect on the incorporation of leucine and isoleucine in the presence of poly U, no case of miscoding with either amino acid was observed.

In other experiments, incorporation of isoleucine and valine was followed in the presence of poly U at 5–40 mM $MgCl_2$. No incorporation of either amino acid in the presence of preincubated ribosomes could be observed.

Cell-free systems from immunized and nonimmunized spleens were tested in the presence of pH 5 fraction from immunized and nonimmunized spleens. All possible combinations of the above fractions were tried and all gave almost identical results. This shows that active antibody formation by the spleen does not influence the miscoding subsequently observed in the cell-free system.

Studies of the same type, but on a smaller scale, were also conducted on cell-free systems containing preincubated ribosomes from either rabbit liver or rabbit reticulocytes. In these systems no miscoding of leucine could be detected in the presence of poly U and 5–25 mM $MgCl_2$.

Discussion.—The work reported here was carried out on a well-defined and thoroughly investigated system. Conditions[32] by which the system could be manipulated in a reproducible and controlled manner minimized experimental error.

Since it was of interest to compare the miscoding properties of spleen with

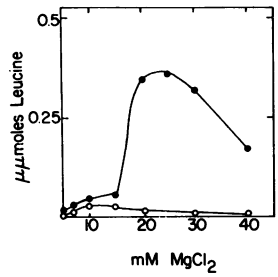

FIG. 5.—Effect of magnesium concentration on transfer of leucine from C^{14}-leucyl-tRNA into protein in the absence and presence of poly U, with the use of preincubated ribosomes. *Open circles*, endogenous incorporation. *Closed circles*, + 15 μg poly U.

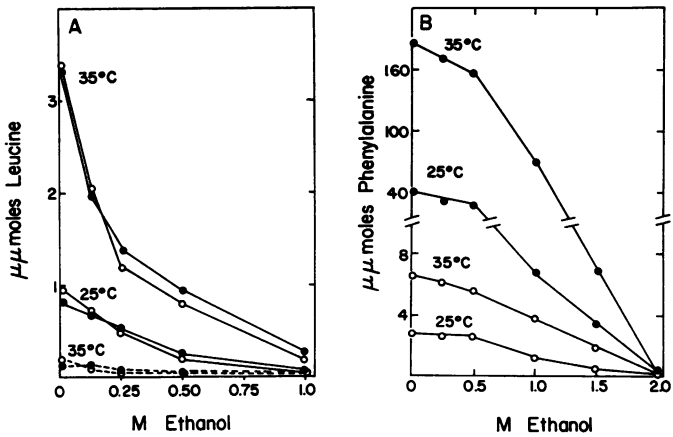

FIG. 6.—Effect of ethanol concentration on the incorporation of C^{14}-leucine and C^{14}-phenylalanine at 25°C and 35°C. (*A*) Incorporation of C^{14}-leucine; (*B*) incorporation of C^{14}-phenylalanine. $MgCl_2$ concentration in all incubation mixtures was 0.012 *M*. *Continuous lines*, nontreated ribosomes. *Dashed lines*, preincubated ribosomes. *Open circles*, endogenous incorporation. *Closed circles*, + 15 μg poly U.

tissues which cannot be triggered to synthesize a specific protein by an external factor, poly U-dependent incorporation of leucine was tested in cell-free systems derived from reticulocytes and from rat liver. As has already been mentioned, the incorporation of leucine was not stimulated by the addition of poly U in either case. While it is tempting to conclude that only certain cells possess the features of miscoding, the possible existence of specific conditions under which poly U would code for leucine in these systems cannot, of course, be entirely ruled out.

As previously noted, whereas isoleucine and valine miscoding has been detected in bacterial systems,[2, 3, 18] none was detected in the spleen system herein tested. Similar to leucine, isoleucine and valine have two U's each in one of their codons.[30, 31] Nevertheless they differ from leucine with respect to their miscoding properties. This suggests that the observed leucine miscoding cannot be attributed entirely to the nucleotide composition of the anticodon segment in the corresponding tRNA molecule. The ratio between the amount of incorporation of leucine and that of phenylalanine in the presence of poly U is a function of

magnesium ion concentration. Even when leucine incorporation is optimal, the ratio is over 1:150 in favor of phenylalanine (Fig. 1). Apparently, a high magnesium concentration permits a certain amount of flexibility in codon-anticodon pairing. However, the degree of flexibility found in the spleen system is much smaller than that of analogous bacterial systems and suggests that translation of the genetic code is more rigorous in mammalian systems.

Cold phenylalanine was routinely added to the incubation mixtures containing C^{14}-leucine. When cold phenylalanine was omitted, a markedly lower level of poly U-dependent leucine incorporation was obtained. This indicates that most of the resultant peptides contain both phenylalanine and leucine.

In summary, it can be said that a specific factor (in this case, magnesium) is able to promote replacement of one amino acid by another (phenylalanine by leucine in the spleen system) in an otherwise normal translational system. The outcome is a peptide with an amino acid composition slightly different from that normally coded for.

In the introduction it was suggested that small differences in amino acid composition between different antibodies could be a result of miscoding. One can visualize a regulatory mechanism involving a factor derived, directly or indirectly, from the immunogen, which could interact with the protein synthetic machinery during γ-globulin biosynthesis, causing slight modifications in the composition of the resulting γ-globulin. The results reported here on an *in vitro* spleen system are consistent with such a proposal.

Summary.—A search for miscoding in a cell-free system derived from spleen, an antibody-producing tissue, was conducted with poly U as template and ribosomes preincubated to remove endogenous incorporation.

In addition to phenylalanine, poly U-directed leucine incorporation was detected. Such poly U-dependent leucine incorporation was observed only at certain magnesium concentrations, markedly different from those optimal for endogenous incorporation or poly U-dependent phenylalanine incorporation. With ribosomes which had not been preincubated, such leucine incorporation was swamped by the endogenous incorporation. Other rabbit tissues examined did not incorporate leucine under these conditions, nor did the spleen preparation incorporate amino acids other than phenylalanine and leucine. Miscoding of leucine could not be enhanced by organic solvents or streptomycin.

The possible significance of miscoding as a control mechanism in antibody-forming cells is discussed.

The author wishes to express his gratitude to Professor S. Yankofsky for valuable suggestions and assistance in the preparation of this manuscript.

* This work was supported by a National Institutes of Health agreement no. 335105, U.S. Public Health Service, Bethesda, Maryland, and by the Délégation Générale de Recherche Scientifique et Technique, France.

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