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*CELL-FREE PROTEIN SYNTHESIS: MESSENGER COMPETITION FOR RIBOSOMES**

BY R. HASELKORN AND V. A. FRIED

DEPARTMENT OF BIOPHYSICS, UNIVERSITY OF CHICAGO

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In a previous communication,[1] it was shown that the over-all kinetics of incorporation of amino acids into polypeptides in a cell-free system from *E. coli* could be accounted for by the following scheme:

$$M + nR \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MR_n \underset{k_4}{\overset{aa\ k_3}{\rightleftharpoons}} MR_n' \underset{aa}{\overset{k_5}{\rightarrow}} MR_n'P$$

in which M = polynucleotide messenger, R = single ribosome, aa = amino acids or their activated intermediates, and P = polypeptide product. When poly U and TYMV-RNA were compared as messengers, the rate of association of poly U with ribosomes was found to be greater than that of TYMV-RNA.[2] These observations have been extended, and in this communication we present results indicating that the association equilibria, as well as forward rates, differ for different polynucleotides. Association of messenger with ribosomes can, under suitable conditions, be the rate-limiting step of amino acid incorporation *in vitro*. An *in vivo* system in which secondary structure of entire messenger RNA molecules is equilibrated, could therefore utilize this feature for controlling the rate of synthesis of particular proteins.

Materials and Methods.—Amino acid incorporation into TCA-precipitable material was measured exactly as described previously.[1] C^{14}-labeled proline and phenylalanine were obtained from New England Nuclear Co., and had specific activities of 205 and 360 mC/mmole, respectively. Poly U and poly C were generously provided by Dr. Stanley Yachnin, who synthesized them from nucleoside diphosphates with *M. lysodeikticus* polynucleotide phosphorylase. These polymers had $S_{20,w}$ = 4.3 S and 4.0 S, respectively, in 0.15 *M* NaCl, 0.01 *M* Tris pH 7.6. Poly C labeled with P^{32} was prepared by Dr. F. Fox in S. B. Weiss' laboratory by using heat-denatured *P. fluorescens* DNA as primer in a reaction catalyzed by *M. lysodeikticus* RNA polymerase. When CTP^{32} is the only nucleotide present, P^{32}-poly C is the product.[3] The product was completely sensitive to pancreatic ribonuclease, formed a helical complex with poly G, and after separation from the DNA primer by centrifugation in Cs_2SO_4, had an ultraviolet absorption spectrum identical with that of poly C made with polynucleotide phosphorylase.[4] Attach-

ment of P^{32}-poly C to ribosomes was studied by zone centrifugation on sucrose gradients, as described previously.[5] P^{32}-labeled TYMV-RNA was prepared as described previously.[5]

Results.—In our previous work,[1] amino acid incorporation stimulated by TYMV-RNA was found to proceed with a lag of 3–4 min at 36°C. Part of this lag could be eliminated by incubation of the RNA with ribosomes in the absence of amino acids, suggesting that in the initial stages of amino acid incorporation *in vitro*, association of RNA with ribosomes was rate-limiting. Furthermore, the kinetic data were qualitatively consistent with an initial *reversible* attachment of RNA to ribosomes. The fact that zone centrifugation on sucrose gradients can be used to demonstrate attachment of poly U and TYMV-RNA to ribosomes indicates that the equilibrium constants for association of these messengers are quite large. On the other hand, Takanami and Okamoto were unable to demonstrate attachment of poly C to ribosomes on a sucrose gradient.[6] Since poly C does stimulate the incorporation of proline, their result suggests that the equilibrium constant for association of poly C with ribosomes is lower than that of poly U or RNA. To test this possibility directly, P^{32}-labeled poly C was prepared as described in *Materials and Methods*, and its association with ribosomes examined on a sucrose gradient. The result is shown in Figure 1. In the absence of ribosomes, poly C is found in

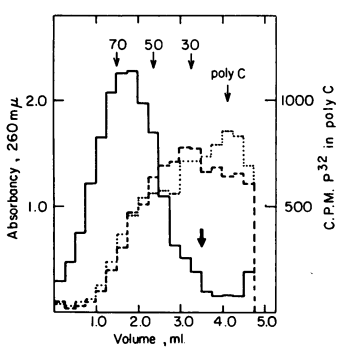

FIG. 1.—Association of P^{23}-poly C with ribosomes. 500 μg ribosomes and 1.5 μg P^{32}-poly C were mixed in 0.01 *M* Mg^{++} in the cold, and centrifuged in the SW39 rotor at 35,000 rpm for 100 min at 10°C. The gradient was 5–20% sucrose in 0.01 *M* Mg^{++}, 0.05 *M* KCl, 0.05 *M* Tris, pH 7.6. The solid line indicates absorbancy, the dotted line P^{32} in poly C. The dashed line indicates P^{32}-poly C in a parallel experiment in which the poly C and ribosomes were incubated for 3 min at 37°C before layering on gradient. Poly C run in the absence of ribosomes is confined predominantly to last three fractions, and no radioactivity travels further than heavy arrow at 25s.

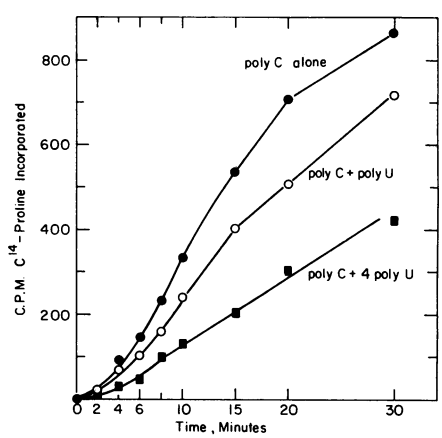

FIG. 2.—Poly U inhibition of the poly C-primed incorporation of proline. Each curve was obtained by taking 0.2-ml aliquots from a 2.0-ml system containing 4 mg ribosomes and 0.48 mg poly C. The reaction labeled poly C + poly U contained, in addition, 18.3 μg poly U; the one labeled poly C + 4 poly U contained 73.5 μg poly U. Incubation was at 25°C.

the region of 5–10s, with absolutely no radioactivity beyond the region indicated by the heavy arrow at 25s. When ribosomes are present, however, the mean excursion of poly C from the meniscus is approximately tripled. The result is only slightly different if the poly C and ribosomes are incubated at 37°C before examination on the gradient. This result means that the equilibrium constant for associa-

tion of poly C with ribosomes is near unity—at least an order of magnitude lower than for poly U.

With this difference in association constants established, it becomes possible to test further the hypothesis that messenger association is rate-limiting in the early stages of amino acid incorporation *in vitro*. When ribosomes are limiting, poly U should be an effective inhibitor of proline incorporation stimulated by poly C. This is shown in Figure 2. With a constant level of poly C, both the rate and extent of proline incorporation are lowered with successive increments of poly U. In the complementary experiment, not shown, poly C had no effect upon phenylalanine incorporation stimulated by poly U.

Since poly U and TYMV-RNA were previously shown to have different forward rates of association with ribosomes,[1] a similar result is expected in the case of poly U-RNA mixtures. Figure 3 shows that, indeed, poly U is an effective inhibitor of

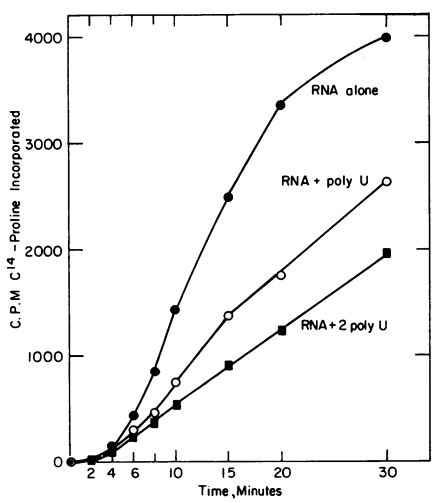

FIG. 3.—Poly U inhibition of the TYMV-RNA-primed incorporation of proline. Each curve was obtained by taking 0.2-ml aliquots from a 2.0-ml system containing 2 mg ribosomes and 290 μg TYMV-RNA. The reaction labeled RNA + poly U contained, in addition, 91 μg poly U; the reaction labeled RNA + 2 poly U contained 165 μg poly U. Incubation was at 36°C.

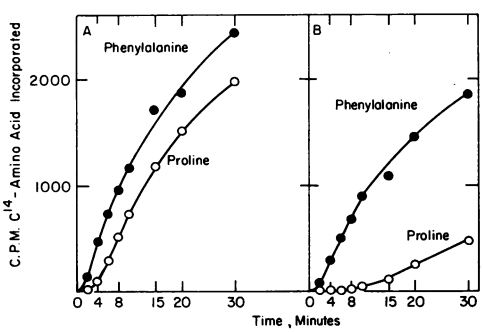

FIG. 4.—Polynucleotide competition for ribosomes. Each curve was determined by taking 0.2-ml aliquots from 2.0-ml systems containing 2 mg ribosomes. In (*A*), two separate reactions contained 165 μg poly C and 580 μg TYMV-RNA, respectively. In (*B*), the two reaction systems contained *both* poly U and TYMV-RNA in these amounts, but C^{14}-proline was added to one, and C^{14}-phenylalanine to the other. Incubation was at 25°C.

proline incorporation stimulated by TYMV-RNA. As with poly C, successive increments of poly U result in a lowered rate and extent of proline incorporation.

The rate of association of RNA with ribosomes is large enough, however, to expect some effect upon phenylalanine incorporation. To study the kinetics more carefully, reactions were run at 25°C instead of 36°C. Figure 4*A* shows the incorporation of phenylalanine and proline when poly U and TYMV-RNA are in separate reaction vessels. Figure 4*B* shows the incorporation of each amino acid, measured separately, in reaction vessels containing the two polynucleotides at the same levels used in the experiment shown in Figure 4*A*. While the rate of incorporation of phenylalanine is depressed slightly, the effect on proline incorporation is striking. We include this figure, despite its redundancy, because it resembles quite closely

the incorporation of valine and histidine stimulated by MS2-RNA.[7] The present work affords an alternative interpretation of the latter experiment which is discussed below.

Having established that poly U present *initially* can interfere with proline incorporation by blocking ribosomal sites for RNA attachment, it was of interest to determine what effect, if any, the addition of poly U would have *after* proline incorporation had begun. Figure 5 shows the result of adding poly U to reactions 2, 6, and 10 min after initiation by TYMV-RNA. At 2 min RNA attachment is incomplete, and poly U depresses the rate of proline incorporation immediately. Addition of poly U at 6 and 10 min has no effect for about 4 min, whereupon the rate of proline incorporation shifts to that characteristic of the RNA: poly U ratio (see Fig. 3). This result suggests first that the poly U inhibition is not due to the creation of a shortage of supernatant. We interpret the difference between poly U addition at 2 min and at 6 min to mean that the initial attachment of TYMV-RNA to ribosomes is rapidly reversible (immediate poly U interference); peptide chain initiation results in conversion to a largely irreversible association. Upon completion of the peptide chain, RNA association again becomes reversible (4 min delay before poly U interferes). If this interpretation is correct, then peptide chain initiation is occurring throughout the course of amino acid incorporation stimulated by TYMV-RNA, as has been shown by Gilbert for the poly U-directed synthesis of polyphenylalanine,[8] and 4 min is the average time needed to complete a peptide chain in this system at 25°C—an order of magnitude lower than the rate at which *E. coli* perform this task.[9]

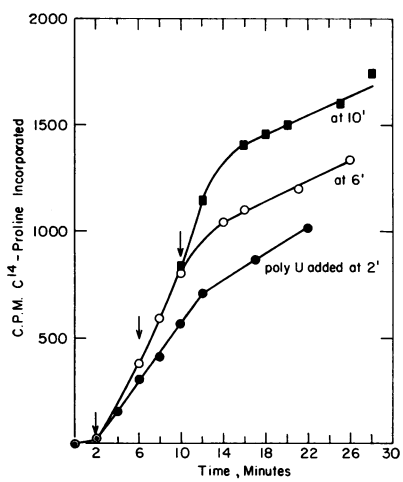

FIG. 5.—Poly U inhibition of TYMV-RNA attachment. Each curve was obtained by taking aliquots from a 2.0-ml system containing 2 mg ribosomes and 580 μg TYMV-RNA. At times indicated by arrows, 165 μg poly U were added. Incubation was at 25°C. Addition of poly U at zero time resulted in a lag of about 8 min, as in Fig. 4*B*.

Before these experiments can be examined further for their relation to protein synthesis *in vivo*, it becomes necessary to look closely at the mechanism of attachment of each messenger. Poly U forms polysomes at 0°C—we therefore consider the poly U attachment to be unrelated to a physiological mechanism for polypeptide chain *initiation*. On the other hand, TYMV-RNA forms monosomes. One interpretation of this result is that TYMV-RNA has a single attachment site for ribosomes. Alternatively, it might have one site with a high association constant for ribosomes, and other sites with much lower constants. To test these alternatives, TYMV-RNA was degraded briefly in alkali, and the association of the resulting fragments with ribosomes examined on a sucrose gradient.

Figure 6 shows that, in a sample which had received an average of seven hydrolytic "hits" per molecule, about 20 per cent of the RNA (by weight) retains the ability of undegraded molecules to associate with ribosomes in the absence of

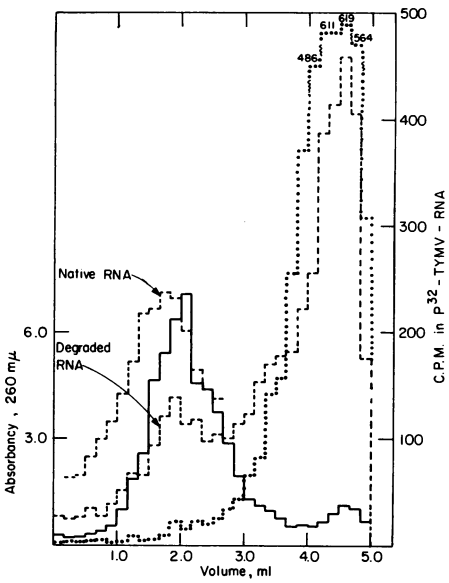

FIG. 6.—Association of alkali-degraded TYMV-RNA with ribosomes. 0.2 ml containing 0.5 mg ribosomes and 24 μg RNA (previously treated with 0.5 *M* KOH at 37°C for 15 sec) were layered on a 5–20% sucrose gradient containing 0.01 *M* Mg^{++}, 0.05 *M* Tris, 0.05 *M* KCl, and run for 60 min at 35,000 rpm at 20°C in the SW39 rotor. The solid line indicates absorbancy, the dashed line radioactivity. The dotted line represents the degraded RNA run alone in a parallel experiment. The same fraction of degraded RNA was bound when the ribosome: RNA ratio was three times greater. The complex of degraded RNA and ribosomes has a sedimentation coefficient about 10s lower than that of native RNA and ribosomes. The total amount of radioactive RNA was greater in the degraded sample than in the native sample depicted. The dashed line for native RNA has been omitted above 3.0 ml.

cofactors other than Mg^{++}. Attempts to extend this type of experiment to yield quantitative data have been complicated by factors discussed below; here we wish to establish only the qualitative point that in TYMV-RNA, in contrast to poly U, the ability to attach to ribosomes is a property of a small region of the native molecule.

Discussion.—The first point we wish to make is that the rate of amino acid incorporation *in vitro* can be limited by the rate of association of messenger with ribosomes. Several lines of evidence can be elicited to support this argument. The first is the interference by poly U with RNA-stimulated incorporation, which is dependent upon the order of addition of the polynucleotides (Fig. 5). The second is the reduction in the lag preceding RNA-dependent incorporation observed when RNA and ribosomes are preincubated in the absence of amino acids.[1] Finally, Szer has found that, whereas poly U stimulates the incorporation of phenylalanine without lag, the poly T-stimulated incorporation of phenylalanine exhibits an initial lag, followed by a period of accelerating rate until the maximal rate of incorporation *exceeds* that of the poly U reaction at the same temperature.[10]

Szer's work also provides the most direct and convincing evidence for the role of secondary structure in governing the association of messenger with ribosomes, since poly U and poly T differ primarily in the degree to which they form self-associated complexes. From the present work, forward rates of association of polynucleotides with ribosomes can be placed in the following order: poly U > TYMV-RNA ≈ poly C. The difference in equilibrium constants for association of the latter two with ribosomes must result from faster dissociation of the poly C-ribosome complex. In our preceding paper, association properties of polynucleotides and degree of secondary structure determined by reactivity toward formaldehyde were correlated[1] (secondary structure: poly U < TYMV-RNA < TMV-RNA ≈ MS2-RNA < ribosomal RNA). Nevertheless, it seems unlikely to us that the gross features of secondary structure revealed by formaldehyde re-

activity or hypochromicity can be a reliable guide to the rate of association with ribosomes. In a sense, the work with synthetic polynucleotides is misleading; whereas with polynucleotides of simple sequence, gross features of secondary structure can be equated with the detailed interactions of individual nucleotides, the same is not true of RNA. It is possible, for example, that MS2-RNA, which is highly ordered, may have short sequences which are "open"—and that these provide the sites for attachment to ribosomes.

Ohtaka and Spiegelman have interpreted their kinetic study of valine and histidine incorporation directed by MS2-RNA in terms of sequential synthesis from a single starting point.[7] Figure 4 shows that a result identical to theirs can be produced by competition between messengers when ribosomes are limiting. On the basis of present information we see no compelling reason to discard either interpretation.

In principle, it would seem a simple matter to settle the question of single or multiple ribosome attachment sites on polycistronic (viral) RNA by comparing the amino acid incorporating activity of native and degraded RNA. Unfortunately, the experimental results are far from clear. Whether the RNA is degraded by heating, as Boedtker and Stumpp have done,[11] or by alkali, as we have done, the incorporation activity of degraded RNA decreases much more rapidly than can be accounted for simply on the basis of separating coding regions from attachment sites. Moreover, mixing experiments indicate that brief alkaline degradation of TYMV-RNA generates inhibitors of amino acid incorporation.[12] Thus, the situation is considerably more complex than we expected; for the present our attention is directed toward the nature of the end groups of native and degraded TYMV-RNA.

Finally, it becomes necessary to ask whether these considerations are relevant to protein synthesis *in vivo*. Regardless of the number of ribosome attachment sites per messenger, differences in the equilibrium constant for association of such sites with ribosomes can only be important if messenger secondary structure is fully equilibrated before association occurs. In our previous paper we suggested that messenger association with ribosomes probably occurred prior to secondary structure equilibration. We envisioned ribosome association occurring while messenger was being synthesized on DNA. This now appears to be the case in the *E. coli* cell-free system.[13] On the other hand, experiments with vaccinia virus-infected HeLa cells indicate that vaccinia messenger RNA exists in a free form prior to association with ribosomes.[14] In such a case the equilibrium properties of the messenger could have a bearing on the control of protein synthesis.

Summary.—Equilibrium constants and forward rates of association of polynucleotides with *E. coli* ribosomes have been found to differ *in vitro*. Messenger association with ribosomes can be the rate-limiting step of amino acid incorporation *in vitro*. The rate of association appears to be correlated with secondary structure of simple polynucleotides; the same may be true of RNA messengers. In contrast to poly U, ribosome attachment sites on TYMV-RNA appear to be restricted to a small region of the native molecule. In certain situations *in vivo* RNA secondary structure may affect the rate of messenger association with ribosomes, and hence the rate of protein synthesis.

Note added in proof: Mr. Peter Moore at Harvard has independently determined that poly C and poly U compete for the same site (s) on ribosomes, and that the affinity of ribosomes for poly U is greater than their affinity for poly C. His measurements consist of sucrose gradient analysis of mixtures of ribosomes and labeled polynucleotides.

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[2] The following abbreviations have been used: poly U, polyuridylic acid; poly C, polycytidylic acid; poly T, polyribothymidylic acid; poly G, polyguanylic acid; TYMV, turnip yellow mosaic virus.

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*SINGLE PEPTIDE DIFFERENCES BETWEEN γ-GLOBULINS OF DIFFERENT GENETIC (Gm) TYPES**

By M. MELTZER, E. C. FRANKLIN,† H. FUDENBERG, AND B. FRANGIONE

NEW YORK UNIVERSITY SCHOOL OF MEDICINE AND UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE

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Genetically controlled polymorphism in human gamma globulins has been intensively studied since its initial recognition by Grubb eight years ago.[1] Similar genetic variations in gamma globulins ("allotypes") have also recently been demonstrated in other mammalian species.[2-4] Reagents to detect the various gamma globulin phenotypes of rabbits, mice, and guinea pigs can be produced by immunizing individuals of one genetic type with gamma globulin of another; allotypic differences are then readily detected by means of precipitating antibodies or by delayed hypersensitivity thus induced. In man, detection of genetic differences between individual gamma globulins has thus far been possible only by inhibition of more complex agglutination systems composed of rheumatoid factors (specific for one or another of the hereditary gamma globulin groups) and inert indicator red cells coated by incomplete antibodies from an individual of an appropriate gamma globulin type. (Rh positive cells coated by incomplete 7S anti-Rh are usually used.[1]) Each of the agglutinating systems thus established is inhibited by normal gamma globulin containing genetic determinants present in the antibody γ-globulin coating