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*A POSSIBLE MECHANISM FOR INITIATION OF PROTEIN SYNTHESIS**

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The process by which the information transcribed from DNA to RNA is translated into the amino acid sequences of proteins must include a precise mechanism for initiating synthesis. Specifically, a particular codon of the messenger RNA must serve as the starting point for synthesis of the polypeptide chain. Current descriptions of protein synthesis, however, do not include an established mechanism for chain initiation. This paper proposes such a mechanism, which is in accord with the well-known behavior of protein-synthesizing systems using natural and synthetic messenger RNA. Additional experimental evidence has been obtained in support of this proposal.

The initiation of polypeptide synthesis requires the fulfillment of two conditions,

either of which may be the point of control. The first is that the ribosome must be attached to the messenger at a site containing the chain-initiating codon; the second is that at least two aminoacyl-sRNA's must be aligned next to each other on the ribosome. The latter is based on current evidence which suggests that the activated carboxyl group of the amino acid is not detached from sRNA except in peptide bond formation.[1-3] Evidence for more than one site for sRNA on the ribosome has been presented by a number of investigators.[3-5]

A mechanism for a highly selective attachment of ribosome to messenger is necessary if control of initiation of synthesis is regulated by fulfillment of the first condition. This selective attachment of the ribosome has been visualized as taking place by two different means. The ribosome binds to the requisite site on the messenger either because it has high affinity for a specific initiating nucleotide sequence, or because other sites on the messenger are not available for binding.[6, 7]

In the light of recent observations that enzymatic formylation of methionyl-sRNA occurs, as well as incorporation of the N-formylmethionine thus formed into polypeptides,[8-10] we have postulated that the control of chain initiation occurs at the second step, i.e., in the alignment of the two aminoacyl-sRNA's on the ribosome rather than in the attachment of the ribosome to the messenger. We have assumed that the interaction of the ribosome and messenger RNA is random, but with natural messenger, the association is not sufficiently stable to allow subsequent alignment of two aminoacyl-sRNA's. Under these conditions, a chain-initiating complex can be formed only with a modified aminoacyl-sRNA which binds to the first of the two sites because of its similarity to peptidyl-sRNA. In other words, a unique triplet which specifies the binding of N-formyl-methionyl-sRNA, for example, to the ribosome would be sufficient to assure proper initiation of protein synthesis.

A corollary to this hypothesis which is applicable to amino acid incorporating systems with synthetic messengers is that if the ribosome-messenger association is sufficiently stable, proper alignment of aminoacyl-sRNA's can take place on the ribosome, and polypeptide synthesis can occur without a modified aminoacyl-sRNA. Synthesis of polyphenylalanine directed by poly U is an example of this case.[11-13] However, as the affinity of the messenger RNA for the ribosome decreases, conditions must be altered in order to stabilize the chain-initiating complex with aminoacyl-sRNA. Amino acid incorporation stimulated by poly A, poly C, and a number of mixed polymers could be considered to be in this category, a higher concentration of magnesium being required for optimal incorporation.[14-17]

If the hypothesis is valid, and a higher magnesium concentration is required in amino acid polymerization with synthetic polymers primarily to enable aminoacyl-sRNA to initiate synthesis, then at a lower concentration of magnesium the system should be dependent on a modified aminoacyl-sRNA with the proper codon. We have conducted tests of this prediction and have found that with low magnesium concentrations and a high temperature, synthesis of polypeptides with synthetic messenger RNA is almost completely dependent on either N-formyl-methionyl-sRNA or peptidyl-sRNA.

Methods.—Preparation of supernatant fraction and ribosomes: E. coli cells, mixed with Macaloid (10 mg/gram of wet cells), were ground with alumina and extracted with 2 vol of a solution containing 0.01 *M* Tris, pH 7.4, 0.01 *M* $MgCl_2$, and 0.001 *M* mercaptoethanol. DNase was added

to the extract (about 1 μg/ml of extracting buffer) prior to centrifugation at 30,000 $\times$ g for 15 min. The ribosomes were then isolated by centrifugation at 105,000 $\times$ g for 5 hr. For most of the experiments, the ribosomes were resuspended and incubated at 37° for 30 min in a solution of 0.01 M Tris, pH 7.4, 0.01 M $MgCl_2$, 0.05 M KCl, and 0.02 M β-mercaptoethanol, centrifuged at 30,000 $\times$ g for 15 min, and then at 105,000 $\times$ g for 2 hr. After a second wash by resuspension in the same buffer and sedimentation at 105,000 $\times$ g, the ribosomes were suspended in a solution containing 0.01 M Tris, 10^{-4} M $MgCl_2$, 0.01 M KCl, and 0.005 M β-mercaptoethanol, and dialyzed overnight in the same buffer. The magnesium concentration of the dialysis medium was increased to 0.01 M about 4 hr before dialysis was terminated. The ribosomes were stored at 0°.

Enough protamine sulfate was added to the supernatant to obtain maximal precipitation of nucleic acids and proteins without loss of amino acid polymerizing activity. Residual protamine sulfate was removed by filtration of the supernatant through a layer of celite. The supernatant fraction was then passed through a Sephadex G-25 column, equilibrated with a solution containing 0.01 M Tris, pH 7.4, 0.05 M KCl, and 0.001 M β-mercaptoethanol, and stored at −20°.

Preparation of N-formyl-C^{14} methionyl-sRNA: Prior to charging the sRNA with C^{14}-methionine and formylation of the methionyl-sRNA, sRNA not specific for methionine was inactivated by charging with C^{12}-methionine and oxidizing the uncharged sRNA with periodate.[18] The C^{12}-methionine was released from the sRNA by incubation for 45 min at 37° and pH 9.5. Activation and formylation of methionine were carried out for 50 min at 37° in a solution containing 0.1 M imidazole, pH 7.2, 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol, 0.0025 M ATP, 1 mg/ml of sRNA, 1.25×10^{-6} M C^{14}-methionine of specific activity 198 μc/μmole, 0.3 mg/ml of the calcium salt of N^5-formyltetrahydrofolate, and 40 μg/ml of supernatant protein. After deproteinization with phenol, the sRNA was precipitated with 2 vol of ethanol and dialyzed. Unformylated methionyl-sRNA was enzymatically deacylated and inactivated by periodate oxidation.[9] Chromatography of the hydrolyzed product on paper in butanol:acetic acid:H_2O (2:1:1) showed that essentially all of the activated methionine was in the form of N-formylmethionine. The formylated methionine was detected on paper by the procedure of Knight and Young.[19] Radioactivity of the chromatograms was measured on a Nuclear-Chicago strip counter.

Preparation of N-formylmethionine: The formylated methionine was prepared by the method of Windus and Marvel.[20]

Preparation of H^3 diphenylalanyl-sRNA: H^3 diphenylalanyl-sRNA was prepared by incubating ribosomes, poly U, H^3 phenylalanyl-sRNA, and supernatant fraction in the absence of GTP. The ribosomes were adsorbed onto Millipore filter, washed, and eluted with a 0.5 M KCl–0.02% triton solution. Following deproteinization with phenol, alcohol precipitation, and dialysis, contaminating phenylalanyl-sRNA was enzymatically deacylated and inactivated by periodate oxidation. Chromatography of a hydrolyzed aliquot on paper in n-butanol:pyridine:H_2O (2:2:1) showed that about 70% of the product was diphenylalanyl-sRNA.

Pronase digestion of the peptides: The reaction mixture was the same as that used in the assay with poly AGU. A 1-ml reaction mixture was incubated for 20 min at 45°. N-ethylmorpholine was then added to approximately 0.4 M and the mixture was heated at 95° for 15 min. The peptides were precipitated and washed two times in cold 10% TCA. After removal of residual TCA with ether, the peptides were suspended in 0.05 M $(NH_4)_2CO_3$ solution, pH 7.4. One-tenth mg of pronase was added and hydrolysis was carried out at 37° for 18 hr. Chromatography was carried out as mentioned for N-formylmethionine.

Assay procedure: The reaction mixture, unless otherwise specified, contained 0.05 M Tris, pH 7.8, 0.008 M $MgCl_2$, 0.04 M KCl, 0.008 M β-mercaptoethanol, 0.001 M GTP, 0.004 M phosphoenolpyruvate, 0.0008 M ATP, 50 μg of sRNA, 5 μg of pyruvate kinase, 19 unlabeled amino acids at 2.4×10^{-5} M with C^{14} phenylalanine, specific activity of 360 μc/μmole, at 5.4×10^{-6} M as the labeled amino acid, 0.25 mg of ribosomes, and about 50 μg of supernatant protein, all in a final volume of 0.25 ml. Incubation was for 15 min. The reaction was stopped by the addition of 5% TCA with poly AGU as messenger, and 10% TCA with poly AU. After being heated for 10 min at 90°, the samples were washed on Millipore filter and counted in a gas flow counter.

Materials.—E. coli, harvested in early logarithmic phase of growth ($^1/_4$ log) was purchased from Grain Processing Corp., Muscatine, Iowa. Macaloid was a product of National Lead Co., Houston, Texas, and calcium salt of N^5-formyltetrahydrofolate (Calcium Leucovorin Injection) was a product of the Lederle Laboratories Division, Pearl River, N. Y. Poly AGU (1:3:10)

was purchased from Miles Laboratories, Elkhart, Indiana. Poly AU (2.6:1) was a generous gift from Dr. S. B. Weiss.

Results.—A first effort was made to obtain an amino acid incorporating system dependent on a formyl donor. Such a system should be stimulated by a synthetic messenger containing the codon for N-formyl-methionyl-sRNA. This was obtained by using an *E. coli* supernatant depleted of sRNA and dialyzable material, and by carrying out polymerization with poly AGU at a suboptimal concentration of magnesium. The magnesium concentration curve for incorporation stimulated by poly AGU (1:3:10) as well as that of poly AU are shown in Figure 1. As observed previously by others, the magnesium concentrations required for optimal incorporation are relatively high. The effect of a formyl donor on incorporation with poly AGU was tested at 0.008 *M* of $MgCl_2$, a level of Mg^{++} far below the optimal required by the system. The results are tabulated in Table 1. Although N^{10}-formyltetrahydrofolate may be the actual formyl donor for the formylation of methionyl-sRNA,[9] the commercially available and more stable N^5-formyltetrahydrofolate was found to be quite effective in our crude *E. coli* extract. Note that 30 μg of N^5-formyltetrahydrofolate increased the polymer-directed incorporation of phenylalanine by almost sixfold.

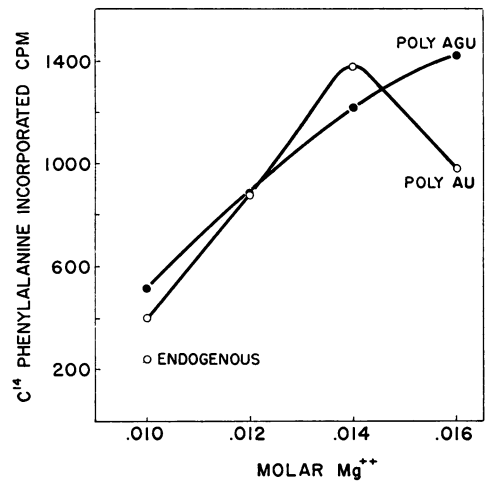

FIG. 1.—Magnesium concentration curves for poly AGU- and poly AU-directed incorporation. The reaction mixture was essentially as described in *Methods*, except that nonpreincubated ribosomes were used and 0.16 *M* NH_4Cl was substituted for KCl. Where specified, 25 μg of poly AGU and 35 μg of poly AU were added. Incubation was at 37°.

The formylated methionyl-sRNA was prepared virtually free of methionyl-sRNA in order to demonstrate that the enhancement of amino acid incorporation was really due to N-formyl-methionyl-sRNA. All other sRNA, including methionine specific sRNA not formylated, was inactivated by periodate oxidation. When N-formyl-methionyl-sRNA was substituted for N^5-formyltetrahydrofolate, the results seen in Table 2 were obtained. The stimulation of incorporation appears to be due to N-formyl-methionyl-sRNA. The amount of labeled methionine incorporated from N-formyl-methionyl-sRNA in the absence of labeled phenylalanine

TABLE 1

STIMULATION OF AMINO ACID INCORPORATION BY N^5-FORMYLTETRAHYDROFOLATE

	Additions	Cpm	Δ
1.	None	161	—
2.	15 μg of N^5-formyltetrahydrofolate	200	39
3.	Poly AGU	343	182
4.	Poly AGU + 15 μg of N^5-formyltetrahydrofolate	959	798
5.	Poly AGU + 30 μg of N^5-formyltetrahydrofolate	1025	864

The composition of the reaction mixture was as described in *Methods* except for the addition of nonpreincubated ribosomes. Where specified, 25 μg of poly AGU were added. Incubation was at 37°.

TABLE 2

STIMULATION OF AMINO ACID INCORPORATION BY N-FORMYL-METHIONYL-sRNA

$MgCl_2$	Additions: C^{14}-phe	Additions: Other	Cpm
0.008 *M*	1. +	None	18
	2. +	Poly AGU	802
	3. +	Poly AGU + 216 μg of N-formyl-C^{14} Methionyl-sRNA	5677
	4. −	Poly AGU + 216 μg of N-formyl-C^{14} Methionyl-sRNA	722
0.016 *M*	5. +	Poly AGU	4455
0.006 *M*	6. +	None	24
	7. +	Poly AGU	54
	8. +	Poly AGU + 216 μg of N-formyl-C^{14} Methionyl-sRNA	2870

The reaction mixture was as described in *Methods* except for indicated changes in $MgCl_2$ concentration. When specified, 50 μg of poly AGU were added. Incubation was at 37°.

TABLE 3

EFFECT OF ELEVATED TEMPERATURE ON THE STIMULATION OF AMINO ACID INCORPORATION BY N-FORMYL-METHIONYL-sRNA

Additions: C^{14}-phe	Additions: Other	Cpm
1. +	None	16
2. +	162 μg of N-formyl-C^{14} methionyl-sRNA	24
3. +	Poly AGU	51
4. +	Poly AGU + 162 μg of N-formyl-C^{14} methionyl-sRNA	3495
5. +	Poly AGU + 216 μg of N-formyl-C^{14} methionyl-sRNA	4589
6. −	Poly AGU + 162 μg of N-formyl-C^{14} methionyl-sRNA	435
7. −	Poly AGU + 216 μg of N-formyl-C^{14} methionyl-sRNA	541

The reaction mixture was similar to that described in Table 2, except that the $MgCl_2$ concentration was kept constant at 0.008 *M*. Incubation was at 45°.

indicates that relatively short peptides are synthesized. This agrees well with the role of N-formyl-methionine as the chain-initiating amino acid in a system with a messenger containing a high frequency of nonsense codons. Doubling the $MgCl_2$ makes the system quite active in the absence of N-formyl-methionyl-sRNA. Although total incorporation is diminished, decreasing the magnesium concentration to 0.006 *M* makes amino acid polymerization almost completely dependent on N-formyl-methionyl-sRNA.

Incubation was carried out at 25° in an effort to make the polymer-stimulated incorporation at 0.008 *M* $MgCl_2$ concentration more dependent on N-formyl-methionyl-sRNA. It was hoped that increased secondary structure of the polymer at the lower temperature would decrease ribosome-polymer interaction. Surprisingly, although over-all incorporation was lower, the system was less dependent on N-formyl-methionyl-sRNA. To keep the results consistent with the hypothesis, it was postulated that lowering the temperature stabilized the chain-initiating complex. If this were the case, then higher rather than lower temperature would be required to make the system more dependent on N-formyl-methionyl-sRNA.

Experiments carried out at 45° seem to bear out this prediction. The results are presented in Table 3. At this elevated temperature the poly AGU-directed incorporation is almost completely dependent on N-formyl-methionyl-sRNA. Again note that significant amounts of labeled methionine are incorporated from N-formyl-methionyl-sRNA. Chromatography of a pronase digest of the peptides synthesized at 45° revealed that no free methionine was liberated even when about 60 per cent of the labeled amino acid was released as formylmethionine.

A kinetic study of incorporation was carried out to rule out the possibility that the failure of polymer alone to stimulate synthesis was due to failure of the labile aminoacyl-sRNA to participate effectively in the slow step of chain initiation at the elevated temperature. The results are presented in Figure 2. The vigorous incorporation with increased magnesium concentration, along with the observation that the polymer-directed incorporation is quite dependent on N-formyl-methionyl-sRNA even at 37°, makes it unlikely that chain initiation is blocked at 45° because of lability of the aminoacyl-sRNA. The methionine activating enzyme is quite active at 45°.

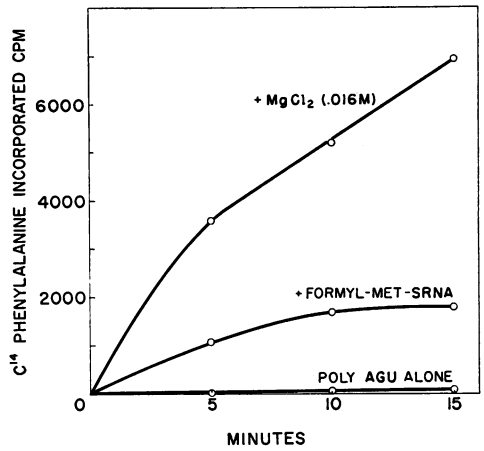

FIG. 2.—Kinetics of poly AGU-directed incorporation. The reaction mixture was similar to that described in Table 2 except for the addition of 25 μg of poly AGU where specified. Incubation was at 45°. Where indicated, 162 μg of N-formyl-C¹⁴ methionyl-sRNA (formyl-met-sRNA) were added.

A preparation of peptidyl-sRNA of H³-phenylalanine containing approximately 70 per cent diphenylalanyl-sRNA was isolated, and its ability to initiate synthesis was examined to obtain supporting evidence for the assumption that N-formyl-methionyl-sRNA is able to initiate synthesis of proteins because of its peptidyl-sRNA-like properties. It is evident from the results presented in Table 4 that the peptidyl-sRNA does enhance the poly AU-directed incorporation of amino acids. The relative stimulation by the peptidyl-sRNA is more accentuated at 45° as in the case of stimulation by N-formyl-methionyl-sRNA. Poly AGU-directed incorporation is also stimulated by the peptidyl-sRNA. Measurement of radioactivity in a gas flow counter with a window excluded all counts due to tritium.

Discussion.—The results of the present study provide evidence for a simple mechanism for the initiation of protein synthesis. With random and weak interaction of ribosome and natural messenger RNA, a unique triplet of the messenger can signal the starting point of polymerization by directing the binding of a modified

TABLE 4

STIMULATION OF AMINO ACID INCORPORATION BY PEPTIDYL-sRNA

Temperature		Additions	C¹⁴-phe incorporated (cpm)
37°	1.	None	48
	2.	50 μg of H³ diphenylalanyl-sRNA	72
	3.	Poly AU	825
	4.	Poly AU + 50 μg of H³ diphenylalanyl-sRNA	1132
	5.	Poly AU + 100 μg of H³ diphenylalanyl-sRNA	1460
45°	6.	None	19
	7.	Poly AU	42
	8.	PolyAU + 200 μg of H³ diphenylalanyl-sRNA	629
37°	9.	None	32
	10.	125 μg of H³ diphenylalanyl-sRNA	58
	11.	Poly AGU	1423
	12.	Poly AGU + 125 μg of H³ diphenylalanyl-sRNA	2410

Conditions were the same as described in *Methods* except for the additions indicated. Where specified, 35 μg of poly AU were added.

aminoacyl-sRNA onto the ribosome. This modified aminoacyl-sRNA, because of its resemblance to peptidyl-sRNA, binds to the peptidyl-sRNA site on the ribosome and initiates synthesis. Under these conditions, aminoacyl-sRNA cannot form the necessary complex, and chain initiation with triplets other than the designated one is prevented.

If the starting point of polymerization is dictated by a single triplet of the messenger RNA, then it follows that this particular triplet should not be repeated elsewhere in the messenger under any circumstances. The case where the translation occurs in proper phase, i.e., the codon being read in the correct group of triplets, need not cause any difficulty, since a unique starting triplet can be designated. The problem is that of avoiding a repetition of the starting codon by an overlap reading of two adjacent triplets. It is perhaps in the resolution of this difficulty that degeneracy in the code plays a vital role.

Degeneracy in the code allows a change in the third place of the codon, and this change appears to be a change of either uracil to cytosine or adenine to guanine.[21] Since in any overlap reading of the codons the last base of the first triplet must be included, a designated triplet can be avoided in all possible overlaps by making the allowed change in the last base of the first triplet. For example, assume that the designated starting triplet is UUG, and that somewhere along the messenger a codon for phenylalanine precedes one for glycine. If the codon UUU for phenylalanine is selected, then the starting triplet is repeated since the sequence will be —U*UU G*GU. However, this can be avoided if the allowed change in the phenylalanine codon is made so that the sequence becomes —U*UC G*GU—.

The stimulation of amino acid incorporation by the peptidyl-sRNA of phenylalanine further supports the view that N-formyl-methionyl-sRNA, because of its similarity to peptidyl-sRNA, binds to the peptidyl-sRNA site on the ribosome and initiates synthesis. The extent of stimulation by the peptidyl-sRNA is surprising when one considers the fact that the codon for the peptidyl-sRNA is not unique. Since both the peptidyl-sRNA and the aminoacyl-sRNA have the same codon, one would expect aminoacyl-sRNA to be a competitive inhibitor of chain initiation, and peptidyl-sRNA to be an inhibitor of chain elongation by the aminoacyl-sRNA. Although part of the stimulation of synthesis by peptidyl-sRNA may be due to the formation of longer peptides sensitive to the method of assay, it is felt that this is not the primary effect. Even in this case, synthesis of the polypeptide is dependent on peptidyl-sRNA for initiation.

The hypothesis provides an explanation for the high concentration of magnesium required for amino acid incorporation directed by synthetic RNA polymers. Even though the polymers have a much greater affinity for the ribosome than does natural messenger RNA, an increased magnesium concentration is still required to start synthesis with aminoacyl-sRNA. Apparently the ribosomes and the enzymes involved in peptide bond synthesis can function over a relatively broad range of magnesium concentration.

A further test for the validity of the hypothesis is to demonstrate that amino acid incorporation with a natural messenger is stimulated not only by the normal modified aminoacyl-sRNA of the system, but by peptidyl-sRNA as well as by a modified aminoacyl-sRNA from another organism. In the first case one would expect synthesis of normal proteins. With peptidyl-sRNA out-of-phase translation can

occur, and nonsense proteins should be the product. Normal proteins should be synthesized with the modified aminoacyl-sRNA from another organism, since a common starting triplet for all organisms is considered more likely from the standpoint of the evolutionary process. However, if the codon is different and the triplet is not excluded from the messenger, incomplete or nonsense protein would be synthesized.

We are currently studying the stimulation of amino acid incorporation in the *E. coli* system with MS 2 RNA as messenger. Work is in progress to isolate a modified aminoacyl-sRNA from rat liver.

Summary.—At low concentrations of magnesium and elevated temperature, the amino acid incorporation directed by poly AGU and poly AU has been shown to be almost completely dependent on N-formyl-methionyl-sRNA or peptidyl-sRNA of phenylalanine. The results support the hypothesis that initiation of protein synthesis is dictated by a unique triplet which directs the binding of a modified aminoacyl-sRNA onto the ribosome. The modified aminoacyl-sRNA, but not aminoacyl-sRNA, forms a chain-initiating complex with natural messenger and ribosome because of its resemblance to peptidyl-sRNA.

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