FORMATION OF INITIATION COMPLEX WITH λmRNA *IN VITRO*

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The product resulting from the *in vitro* transcription of native λDNA by purified *E. coli* RNA polymerase does not stimulate total amino acid incorporation although it can program ribosomes for the first peptide bond synthesis in the presence of the three initiation factors A, B and C.

1. Introduction

The formation of polypeptides in cell free systems from *Escherichia coli* is dependent on the addition of either DNA or RNA with messenger activity and some factors responsible for the transcription and translation. While DNA from T-even bacteriophage appears to be an active primer in a coupled system from *E. coli*, bacteriophage λDNA, usually causes little stimulation of amino acid incorporation under standard conditions [1, 2]. The same difference appears to the template activities of the corresponding *in vitro* synthesized RNAs, although synthetic T_4mRNA, once purified, is usually less active than the DNA bound T_4mRNA chains [3].

Because our laboratory is actually engaged in studying the control of gene expression during bacteriophage λ development, we have been interested to further explore the translation of λmRNA in cell free systems. The inability to obtain the synthesis of polypeptides on λmRNA synthesized *in vitro* could be related with some defect in λmRNA–ribosome interaction or to some other events interfering with the initiation or elongation of protein synthesis on this phage specific template. In order to study these possibilities, we have specially followed the formation of complex by *E. coli* 70 S ribosomes, λmRNA and fMet-tRNA, in the presence of various initiation factors.

The data presented show that λmRNA made *in vitro* efficiently binds to 70 S ribosomes and that this reaction requires the presence of the 3 initiation factors together with *N*-formylmethionyl-tRNA. The bound fMet-tRNA can react with puromycin to form fMet-puromycin but acid-precipitable polypeptide products cannot be synthesized, suggesting that some step in elongation of the polypeptide chain does not take place.

2. Materials and methods

In vitro synthesis of labelled λmRNA (^{3}H-λmRNA) was effected in the following conditions: 40 μg of λDNA (derived from strain $C_{600}\lambda C_{I\ 875}$) and 20 units of RNA polymerase; tris-HCl 0.02 M, pH 8.0; $MnCl_2$ 0.001 M; $MgCl_2$ 0.008 M; ^{3}H-GTP 0.008 M (30 μCi per mole); 3 other nucleoside triphosphates 0.008 M; and 2-mercaptoethanol 0.01 M, were mixed in the cold in a total volume of 2.5 ml. The mixture was incubated for 20 min at 37°, and 10 μg/ml of DNase was added and then incubated for another 15 min. The mixture was heated for 3 min at 95° and chilled immediately. After adding one volume of 0.5% of SDS (sodium dodecyl sulfate) in 2 × SSC and two volumes of phenol saturated with 2 × SSC, the mixture was shaken at 65° for 2–3 min and then at 4° for 30 min. After centrifugation, the aqueous layer was taken up and treated again with phenol. Then the final aqueous

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phase was dialysed for two hours in the cold against successively, 2 × SSR solution containing 0.2% SDS, 2 × SSC containing 0.1% SDS and, finally, against 2 l of 0.02 M tris-HCl (pH 8.0) solution containing 0.01 M 2-mercaptoethanol [9]. The ribosomes and initiation factors were prepared from *E. coli* MRE 600 following the method described elsewhere [4]. Factor C was purified according to Revel et al. [5].

Binding of ^{3}H-λmRNA to 70 S ribosomes was carried out in tris-HCl 0.05 M, pH 7.6; NH_4Cl 0.1 M; Mg-acetate 0.005 M; GTP 0.005 M; 2-mercaptoethanol 0.005 M, in a final volume of 0.48 ml. The reaction mixture was incubated for 5 min at 37° and layered on the top of 10 to 30% (w/v) of glycerol gradient containing tris-HCl 0.02 M, pH 7.6; Mg-acetate 0.005 M; NH_4Cl 0.1 M and dithiothreitol 0.001 M. After centrifugation for 110 min at 38,000 rpm in a Spinco (rotor SW 39), fractions were collected from each gradient and the radioactivity determined after filtering each fraction through membrane filter (Sartorius 0.6 μm) and washing with the buffer used for the incubation.

Labelled and non-labelled fMet-tRNA were a gift from Dr. J.Dondon. DNA (T_4DNA or λDNA) dependent amino acid incorporation was measured in 0.125 ml of incubation mixture containing tris-HCl 5 μM pH 7.6; $MgCl_2$ 1.9 μM; NH_4Cl 24 μM; 2-mercaptoethanol 0.75 μM; ATP 0.25 μM; GTP, CTP and UTP 0.05 μM; each; ^{14}C-valine 0.01 μM and 19 cold amino acids 0.025 μM; pyruvate kinase 5 μg; T_4DNA or λDNA 15 μg; S-100 fraction 0.5 A_{260} units; purified RNA polymerase 10 units; ribosomes 150 μg; factor A 8 μg; factor B 16 μg and factor C 2 μg. After 30 min of incubation at 37° the reaction was stopped with 5% TCA and the samples heated at 90° for 20 min, filtered and counted in a Nuclear Chicago scintillation counter using a PPO–POPOP–toluene mixture.

Table 1
Formation of the initiation complex between *in vitro* synthesized labelled λmRNA and T_4mRNA and ribosomes from *E. coli* MRE 600.

Factor addition	Radioactivity found in the complex (cpm)	
	^{3}H-T_4mRNA	^{3}H-λmRNA
none	125	208
C	175	357
C + A	534	515
C + B	392	–
C + A + B	800	711
without fMet-tRNA	29	30

Incubation mixture consisted of the following components: tris-HCl 0.05 M, pH 7.6; NH_4Cl 0.1 M; Mg-acetate 0.005 M; GTP 0.005 M; 2-mercaptoethanol 0.005 M; 2 μg of factor C; 8 μg of factor A; 16 μg of factor B; 0.940 A_{260} of 70 S ribosomes (*E. coli* MRE 600); fMet-tRNA non-labelled 1.1 A_{260} and ^{3}H-T_4mRNA or ^{3}H-λmRNA (^{3}H-T_4mRNA, 16,000 cpm and ^{3}H-λmRNA, 15,600 cpm put in for each case). The technique of Nirenberg and Leder was used [6].

3. Results and discussion

The first step in the synthesis of polypeptide chains is the interaction between mRNA, 30 S ribosomal subunits and fMet-tRNA in the presence of the initiation factors. This complex can be retained on a membrane filter [6]. We have tried to detect this initiation complex with synthetic λmRNA, 70 S ribosomes and initiator tRNA, plus a mixture of purified initiation factors A, B and C. *In vitro* synthesized T_4mRNA was used as a control. The results shown in table 1 indicate that ^{3}H-λmRNA can readily bind to 70 S ribosomes and that the formation of this complex is entirely dependent upon the combined addition of initiator tRNA plus 3 factors. Formation of the complex is stimulated much more by the three factors together than with factor C alone or by any of the binary

Table 2
Formation of fMet-puromycin in the presence of λmRNA.

Complex mixture*	^{3}H-fMet-tRNA recovered in ethyl acetate extracts (cpm)
with λmRNA	501
with poly AUG	1444

* Complete mixture: tris-HCl, 0.05 M, pH 7.8; NH_4Cl, 0.08 M; Mg-acetate, 0.005 M; 2-mercaptoethanol, 0.007 M; GTP, 0.005 M; factor C, 2 μg; factor A, 8 μg; factor B, 16 μg; puromycin 50 μg per sample. 0.960 A_{260} of ribosomes were used. Unfractionated ^{3}H-fMet-tRNA was used (Sp. act. 350 cpm/pmole). fMet-puromycin products were extracted by ethyl acetate. Results are expressed after deducing the background without exogenous mRNA (706 cpm). Control without factors, 404 cpm.

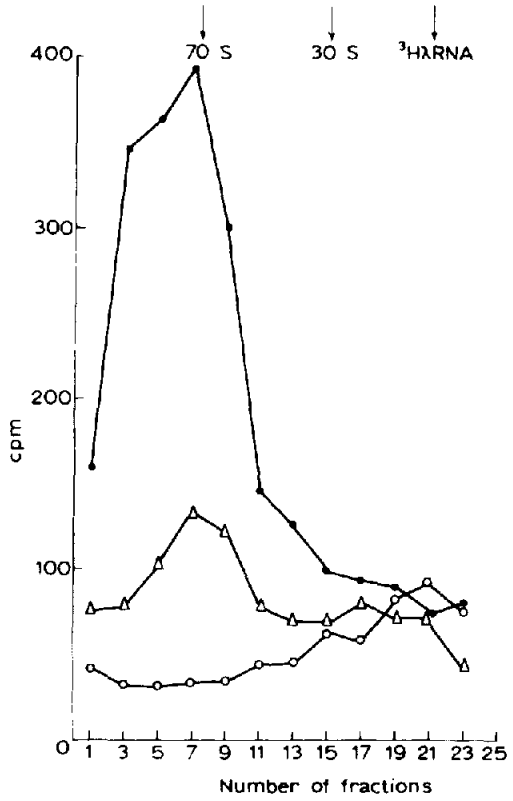

Fig. 1. Sedimentation pattern of the initiation complex formed between the ^{3}H-λmRNA, 70 S ribosomes and initiation factors (A, B and C). ●—●—● complete system indicated in table 1; △—△—△ in the presence of fMet-tRNA but without initiation factors; ○—○—○ in the presence of 3 factors together but without fMet-tRNA.

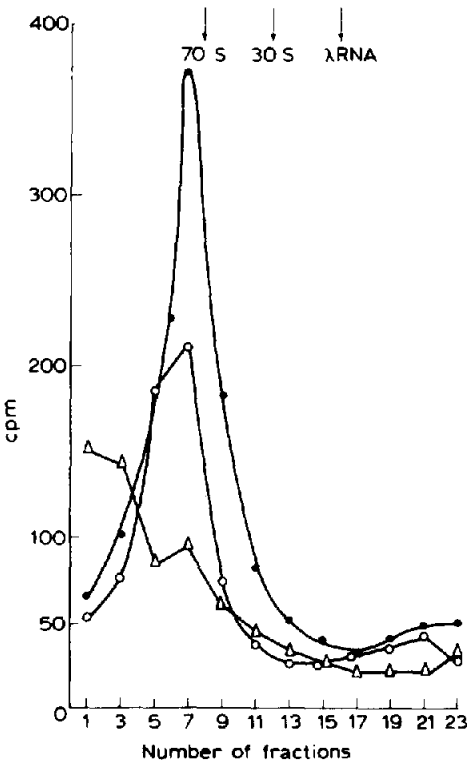

Fig. 2. Formation of the initiation complex followed by labelled ^{3}H-fMet-tRNA in density gradient centrifugation. The conditions are given in the legend of the fig. 1. ●—●—● complete system; ○—○—○ with 3 factors but without λmRNA; △—△—△ with λmRNA but without initiation factors.

combination of factors tested. The extent of λmRNA binding in the complete system is roughly similar to T_4mRNA on basis of the total radioactivity input.

We have also followed the formation of initiation complex by centrifugation. As can be seen in fig. 1, bound ^{3}H-λmRNA sediments with 70 S ribosomes in glycerol gradients, whereas in the absence of initiation factors the radioactivity attached to 70 S ribosomes is very small. This fact indicates that our preparation of NH_4Cl washed ribosomes is practically devoid of initiator factors. No significant radioactivity is bound to 70 S ribosomes in the absence of fMet-tRNA even in the presence of the initiation factors. The same experiment was also repeated using cold λmRNA synthesized *in vitro* and radioactive initiator tRNA (fig. 2). Radioactivity of this tRNA is again localized in the 70 S ribosomal region of the gradients, dependent upon the initiation factors, but in this case, a certain amount of radioactivity is detectable on the ribosome peak even in the absence of λmRNA. This results reflects presumably the contamination of small amounts of endogenous bacterial mRNA in the ribosome preparation.

The above results indicate that *in vitro* transcribed λRNA participates as efficiency in the formation of an initiation complex as does T_4mRNA. Particularly worthy of notice is the stimulation by factor B which is known to be specifically required to initiate translation of natural messenger RNAs [7]. That the complex obtained with λmRNA represents a true reactive step

Table 3
Protein synthesis in coupled RNA polymerase, ribosomal systems with T_4 and λDNA, or in the presence of synthetic T_4 or λ specific mRNA.

Incorporation of ^{14}C-valine[a] (pmoles per 150 μg ribosomes)			
T_4DNA dependent[b]	λDNA dependent[b]	T_4mRNA[c]	λmRNA[c]
148	<1	5	1

[a] Background for coupled synthesis is 12 pmole and has been subtracted.
[b] Conditions were described in Materials and methods.
[c] 2.15 μg of T_4mRNA and 4.25 μg of λmRNA were used respectively. Values in the absence of T_4mRNA were subtracted (6 pmole).

in polypeptide synthesis is supported by the fact that bound ^{3}H-fMet-tRNA readily reacts with puromycin (table 2). Although this latter result indicates that λmRNA translation with *E. coli* ribosomes can at least proceed until the stage of first peptide bond formation, data shown in table 3 indicate that this RNA is totally unable to stimulate total amino acid incorporation into a TCA precipitable material, confirming earlier results [2, 3].

λmRNA used in these experiments presumably corresponded to early gene transcripts [8]. A search is presently undertaken to find if short TCA soluble peptides can actually be synthesized on this phage specific RNA template. The mechanism responsible for the lack of continuation of polypeptide synthesis is unknown. It might be related to a very high degree of secondary structure of the synthetic template involved or less likely to the need for some phage specific translation factors. These questions are under study.

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