INDEPENDENT INITIATION OF TRANSLATION OF TWO BACTERIOPHAGE f2 PROTEINS

Harvey F. Lodish
Medical Research Council Laboratory of Molecular Biology
Hills Road
Cambridge, England

and

Massachusetts Institute of Technology*
Department of Biology
Cambridge, Massachusetts 02139
U.S.A.

Received August 8, 1969

SUMMARY

We describe an E. coli ribosome system which forms only the first peptide bond of f2 proteins. Intact f2 RNA directs formation of the amino-terminal dipeptides of both the coat and maturation protein, a result showing directly that ribosomes can independently attach to two sites on the f2 RNA. A specific fragment of f2 RNA directs formation also of the first peptide bond of the RNA polymerase.

INTRODUCTION

The single-stranded RNA from small bacteriophages such as f2 codes for only three known proteins: coat protein, maturation or "A" protein (a minor component of the virus particle) (1) and the phage RNA polymerase (2,3). When f2 RNA is used as messenger in a cell-free system from Escherichia coli, the predominant reaction product is the virus coat protein (4). f2 RNA directs the synthesis as well of the two other virus proteins: RNA polymerase, made at 30% the amount of coat protein (5,6,7,8), and the maturation protein, made at one to three percent of the coat (7,8).

---

* present address

Using specific fragments of f2 RNA we could show that each of the f2 genes can be translated independently of the synthesis of the other proteins (7). It was also apparent that even the intact f2 RNA is not translated sequentially from one end, as predicted in some models of messenger RNA translation (9). Rather, the synthesis of the f2 coat and maturation proteins proceeds simultaneously and independently, although at markedly different rates. However, translation of the polymerase gene follows and is dependent on synthesis of at least part of the coat protein (7,24-27). These results suggested a model of f2 translation (7,10). E. coli ribosomes attach independently, although at different rates, to two sites on the f2 RNA - - at the site for initiation of translation of the coat and maturation proteins. Ribosome attachment to the beginning of the polymerase gene, however, would be dependent on a change in the RNA tertiary structure which we postulate occurs during translation of the coat gene.

In the current work we test some of the predictions of this model using a ribosome system which directs formation of only the first peptide bond of the f2 proteins. Under conditions where degradation of intact f2 RNA is minimal, E. coli ribosomes do direct formation of the initial peptide bond of the coat and maturation proteins, but not of the polymerase protein.

RESULTS AND DISCUSSION

The composition of the reaction mixture is contained in the legend to Fig. 1. Except for the supernatant enzymes, all of the components necessary for protein synthesis (11) are added. As all f2 proteins begin with N-formyl methionine, we used N-formyl (^{35}S) methionyl tRNA to label the peptides formed. The reactions were incubated at 37°C for up to 10 minutes. After mild alkaline digestion to hydrolyze the peptidyl-tRNA linkage, the radioactive products were analyzed by paper ionophoresis at pH 3.5. In a reaction without added viral RNA (Fig. 1e), one finds

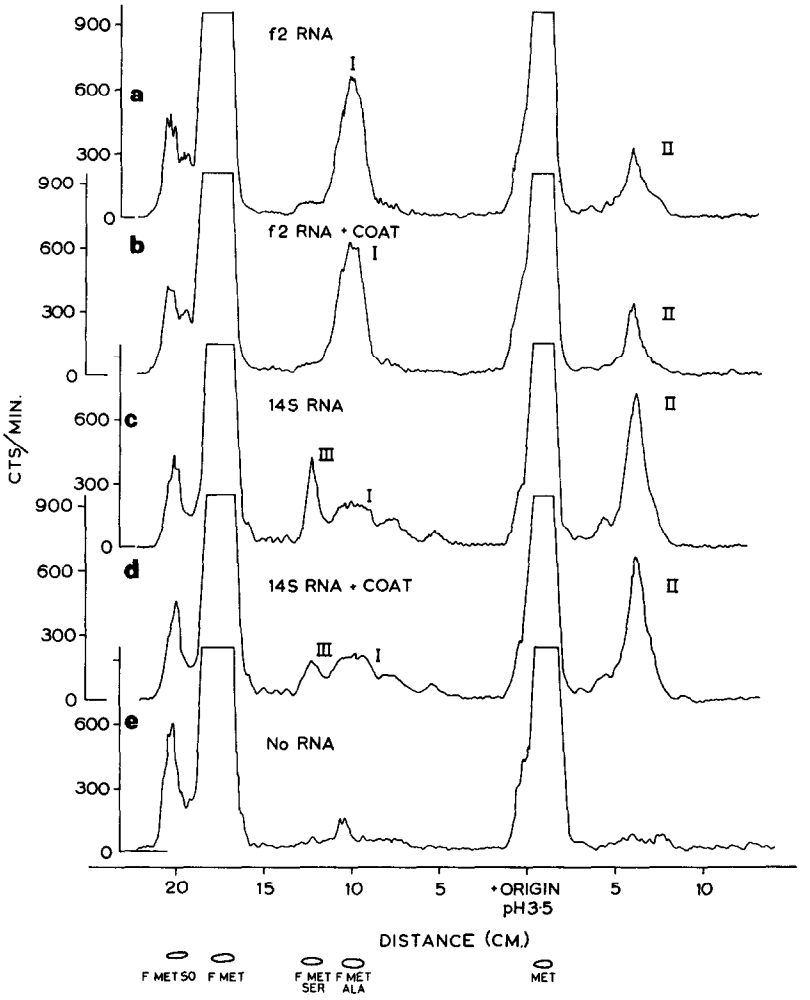

Fig. 1. Formation of N-formylmethionyl aminoacyl tRNA.
(a) directed by 27S f2 RNA.
(b) directed by a complex of 27S f2 RNA and coat protein.
(c) directed by 14S RNA.
(d) directed by a complex of 14S RNA and coat protein.
(e) without added RNA.

Reaction mixtures (0.1 ml) contained, per ml: 100 μmoles Tris Acetate, pH 7.2; 50 μmoles KCl, 5 μmoles $MgCl_2$; 0.3 μmoles GTP; 400 μg *E. coli* tRNA charged with (^{35}S) methionine (5000 mc/mM; about 70% of methionine tRNA formylated) and 19 non-radioactive amino acids; 19 A_{260} units *E. coli* ribosomes; and 200 μg initiation factors. Phage RNA's were added at 300 μg/ml and coat protein (22) at 96 μg/ml, equivalent to 20 molecules per molecule of f2 RNA. Ribosomes and crude initiation factors were prepared from *E. coli* MRE600 cells by the procedure of Anderson *et al.* (23), except that the ribosomes were pelleted 3 times in 0.06M NH_4Cl solution before the initiation factors were removed from the ribosomes in 2.0M NH_4Cl. Incubation was for 3 minutes at 37°C. 0.5 ml 0.4M triethylamine was added and the reaction incubated at 37°C for one hour. After lyophilization the samples, together with marker peptides, were analyzed by paper ionophoresis at pH 3.5. Radioactivity was measured in a Packard 7201 strip scanner at 0.5 cm/min, with a 1000 cts/min scale. Marker peptides were visualized with the platinic iodide stain.

only radioactive methionine, formyl methionine, and formyl methionine sulfoxide, all of which are derived from the tRNA added to the reaction. Addition of 27S f2 RNA results in formation of two new radioactive peptides, I and II (Fig. 1a).

Identification of products. Peptide I is N-formyl methionyl alanine (FMet Ala), the dipeptide at the amino terminus of the f2 coat protein (12). I was inseparable from authentic FMet Ala by paper ionophoresis at pH 3.5, 4.5, and 6.5, and in the paper chromatography in the solvent system described previously (7). After treatment of I with dilute HCl to remove any formyl residue, it yielded a radioactive peptide inseparable from the marker methionyl alanine. Furthermore, in one experiment tRNA charged with (^{35}S) methionine and (^{3}H) alanine was used, and the peptide I formed contained equimolar amounts of (^{35}S) and (^{3}H) radioactivity.

Peptide II is N-formyl methionyl arginine, the dipeptide found at the amino terminus of the f2 maturation protein (10,13). It is formed in 15% the amount of I. In experiments in which tRNA charged with (^{35}S) methionine and different (^{3}H) amino acids was used, radioactivity from only (^{3}H) arginine was recovered in peptide II. Figure 2 shows that II has an electrophoretic mobility identical to that of the tryptic peptide (E) containing formyl (^{35}S) methionine which is found at the amino terminus of the f2 maturation protein synthesized in vitro. Likewise, deformylation of peptide II and tryptic peptide E yielded peptides with identical ionophoretic mobilities (Fig. 2).

These results suggest that, with intact f2 RNA, E. coli ribosomes direct formation of the first peptide bond both of the f2 coat and maturation proteins. This implies that ribosomes can attach to at least two sites on a messenger RNA. However, two ribosomes might not be able to attach simultaneously to the same messenger; possibly initiation of one protein precludes ribosome attachment to the beginning of the other gene. Note that with 27S f2 RNA no N-formyl

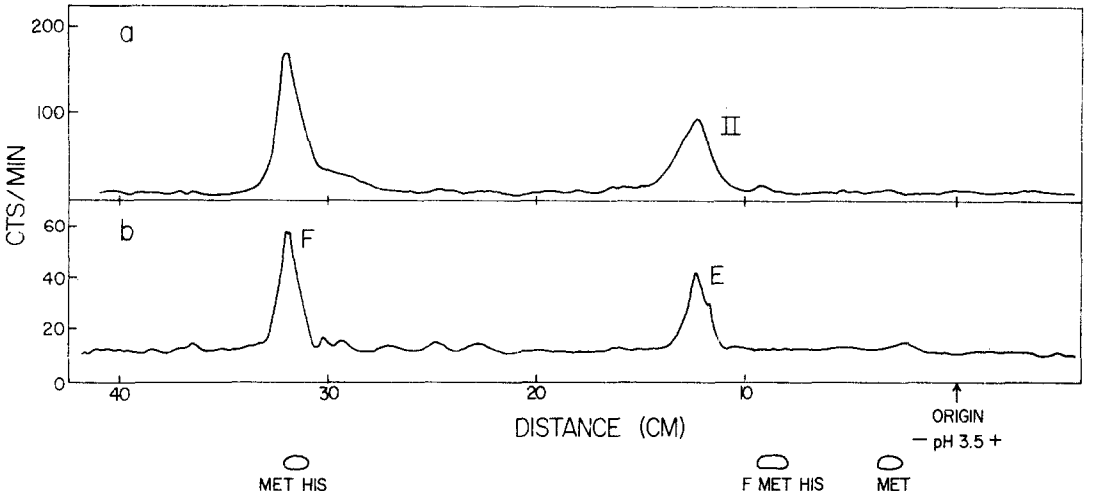

Fig. 2. Identification of FMet peptide II.
(a) Peptide II treated with HCl.
(b) Mixture of tryptic peptides E and F from the amino terminus of the maturation protein.

Peptide II (Fig. 1a) was eluted from the paper, and digested with 0.25 N HCl:dioxane (1:1) at 37°C for 16 hours. Under these conditions about 60% of the formyl residue is removed from typical FMet peptides. Peptides E and F are labeled with (^{35}S) methionine; E is the formylmethionine-containing peptide from the amino terminus of the maturation protein, and F is its deformylated derivative (ref. 7,8). The two samples were analyzed by paper ionophoresis at pH 3.5 on adjacent regions of a sheet of 3MM paper.

---

methionyl serine (FMet Ser) is formed (Fig. 1a). Since this is the amino terminal sequence of the f2 RNA polymerase (7), we can conclude that ribosomes cannot directly initiate synthesis of RNA polymerase, a result which is also consistent with our model of f2 RNA translation.

Intactness of f2 RNA. Figure 3 shows that over 95% of the f2 RNA used in these reactions sedimented with an S_{20} of 27, characteristic of whole f2 RNA molecules. When labeled RNA was used and reisolated after three or ten minutes reaction, still over 90% of the material sedimented at 27S. Since both the binding to ribosomes of FMet tRNA (15) and the formation of the two FMet dipeptides (unpublished observation) were linear with time for at least 10 minutes at 37°C, it

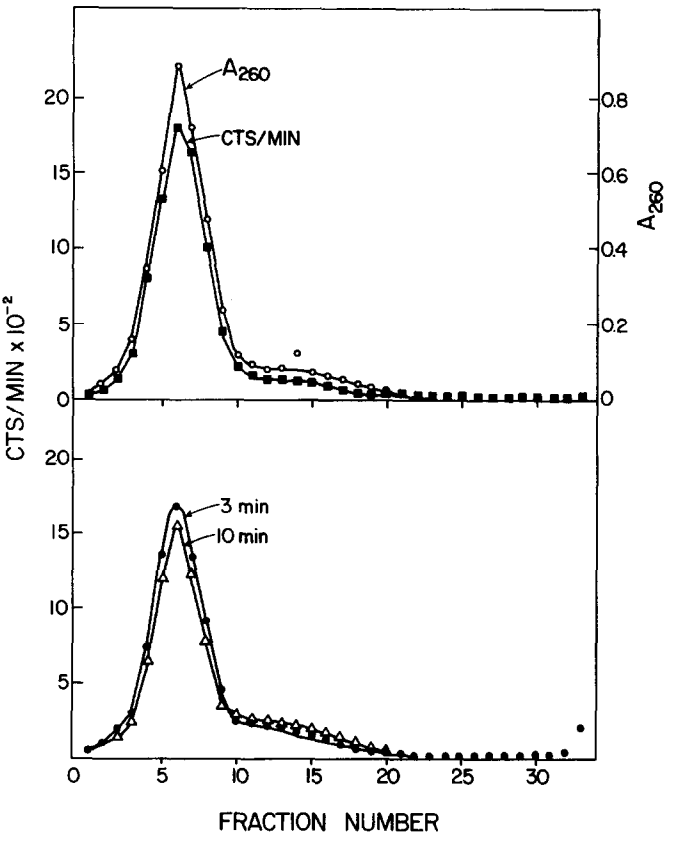

Fig. 3. Sucrose gradient analyses of (^{3}H) f2 RNA.
(Top) Sample of RNA added to reaction mixture.
(Bottom) RNA reisolated from the reaction after 3 and 10 minutes incubation.

27S f2 RNA containing (^{3}H) uracil (approximately 4.4×10^4 cts/min/mg RNA) was used. In the bottom panel, 0.5 ml aliquots from a reaction of composition identical to that of Fig. 1 were chilled at the indicated times, and immediately extracted with an equal volume of water-saturated phenol. After precipitation with ethanol, the RNA was dissolved in 0.1 ml water and layered on a 4.8 ml 5-20 percent sucrose gradient containing 0.10M NaCl; 0.05M Tris, pH 7.5; and 0.5% sodium dodecyl sulfate. Centrifugation was for 150 minutes at 50,000 rpm, 20°C, in the SW65 rotor of the Model L-2 ultracentrifuge. Total radioactivity was determined on each fraction by drying an aliquot on a fiberglass filter and counting in a scintillation counter. Centrifugation is from right to left.

---

appears unlikely that the synthesis of either FMet Ala tRNA or FMet Arg tRNA is directed exclusively by some fragment of f2 RNA produced during the reaction. However, since the amount of FMet Arg tRNA produced is only about 5% that of the f2 RNA bound to ribosomes, it

is possible that only some minor component in the added f2 RNA does direct the synthesis of FMet Arg tRNA.

Table 1

Synthesis of N-formyl (^{35}S)-methionyl aminoacyl tRNA directed by f2 RNA

Reaction	μμmoles/0.1 ml (^{35}S) FMet		
	Bound to Ribosomes	In formylmethionyl-alanine	In formylmethionyl-arginine
Complete	3.19	0.28	0.04
Minus f2 RNA	0.27	0.03	0.01
Minus initiation factors	0.32	0.03	0.01
Minus GTP	0.25	0.03	0.02
Minus GTP, plus GMPPCP	1.38	0.03	0.01
No alkaline hydrolysis	—	0.02	0.01

Reaction mixtures (0.2 ml) are described in the legend to Fig. 1. GMPPCP was used at 0.3 μmoles/ml. After 3 minutes incubation at 37°C, a 100 μl aliquot from each was diluted into 5 ml of ice-cold buffer (0.1M Tris, pH 7.2; 0.05M KCl; 0.005M $MgCl_2$) and filtered through a prewetted Millipore filter (HAWP, 2.4 cm). After washing the filter with four 5 ml batches of buffer, the filters were dried and counted; the radioactivity represents (^{35}S) FMet tRNA bound to ribosomes (23). The remainder of the reaction was digested with triethylamine and analyzed as in Fig. 1.

Requirements. The requirements for the formation of FMet dipeptides are shown in Table 1. f2 RNA, GTP and crude initiation factors are required both for binding to ribosomes of FMet tRNA and formation of FMet Ala tRNA and FMet Arg tRNA. Guanosine methylene diphosphonate (GMPPCP), an analog of GTP which cannot be hydrolyzed to GDP (14), can replace GTP in the binding of FMet tRNA to ribosomes, but not in the formation of FMet aminoacyl tRNA. This is in agreement with the inability of GMPPCP to substitute for GTP in the formation of N-formyl methionyl puromycin in a reaction similar to that used here (15). No

FMet Ala or FMet Arg is observed if the reaction is not hydrolyzed with alkali, a result showing that all dipeptides formed are still attached to a tRNA.

Apparently our reaction system is deficient in the G (or translocation) enzyme of protein synthesis (11,16,17), for we observe no tripeptides (e.g. formyl methionyl alanyl serine from the f2 coat protein) or other oligopeptides. This interpretation is supported by the observation that fusidic acid, a drug which specifically inhibits the G translocation reaction (16,18) has no effect either on the binding to ribosomes of FMet tRNA or on the formation of FMet Ala tRNA or FMet Arg tRNA.

14S RNA. Finally, we consider the ability of a specific fragment of f2 RNA, 14S RNA, to initiate protein synthesis. We have previously shown that 14S RNA directs synthesis of maturation and polymerase protein, but only a very small amount of coat protein, presumably because it does not contain the f2 coat protein gene (7,19). In contrast to 27S f2 RNA, 14S RNA directs synthesis of polymerase in the absence of synthesis of f2 coat protein. This suggests that, in 14S RNA but not 27S f2 RNA, ribosomes can attach directly to the site for initiation of polymerase synthesis. Consistent with this explanation, Fig. 1c shows that 14S RNA does direct the formation of peptide III, which is FMet Ser (the dipeptide at the amino terminus of the polymerase), as well as of peptide II, FMet Arg. The small amount of peptide I produced may represent residual initiation of coat protein synthesis or, alternatively, initiation at some artifactual site on 14S RNA which does not correspond to any known f2 protein.

To show that the FMet Ser tRNA produced does indeed derive from the amino terminus of the polymerase, we take advantage of the finding that f2 coat protein binds to f2 RNA in such a manner as to inhibit specifically synthesis of RNA polymerase, but not of coat and maturation protein

(7,20,21). Figure 1d shows that addition of 6 moles of coat protein per mole of 14S RNA specifically inhibits formation of FMet Ser tRNA, but has no effect on synthesis of FMet Arg tRNA or FMet Ala tRNA (Fig. 1).

Taken another way, these results show that coat protein binds to f2 RNA in such a manner as to inhibit initiation of synthesis of RNA polymerase. Presumably the coat protein binding site is near, if not identical to, the site for initiation of polymerase synthesis.

The results also show that the inability of 27S RNA to direct formation of FMet Ser tRNA is not due to some deficiency in the assay system but rather that E. coli ribosomes actually cannot attach to the site for initiation of polymerase synthesis. One prediction of our model of f2 translation is that it should be possible to modify the tertiary structure of 27S f2 RNA so that it can directly initiate synthesis of RNA polymerase. The system for formation of FMet aminoacyl tRNA's reported in this paper should facilitate the detection of such changes in RNA tertiary structure (10).

ACKNOWLEDGMENT

This research was supported by grant #AI-08814 from the U.S. National Institutes of Health and by a postdoctoral fellowship from the American Cancer Society. The superb technical assistance of Miss Elaine Holgate is appreciated.

REFERENCES

1. Steitz, J. A. (1968). J. Mol. Biol. 33, 923.
2. Horiuchi, K., Lodish, H., and Zinder, N. (1966). Virology 28, 438.
3. Gussin, G. N. (1966). J. Mol. Biol. 21, 435.
4. Nathans, D., Notani, G., Schwartz, J. H., and Zinder, N. D. (1962). Proc. Nat. Acad. Sci., U. S. 56, 1844.
5. Vinuela, E. Algrananti, I., and Ochoa, S. (1967). Europ. J. Biochem. 1, 3.
6. Capecchi, M. (1966). J. Mol. Biol. 21, 173.
7. Lodish, H. F. (1968). Nature 220, 345.
8. Lodish, H. F., and Robertson, H. (1969). J. Mol. Biol., in the press.

9. Martin, R. G., Whitfield, H., Berkowitz, D., and Voll, M. J. (1966). Cold Spring Harbor Symp. Quant. Biol. 31, 215.
10. Lodish, H. F. (1969). Cold Spring Harbor Symp. Quant. Biol. 34, in the press.
11. Lipmann, F. (1969). Science 164, 1024.
12. Webster, R. E., Engelhardt, D., Zinder, N. D., and Konigsberg, W. (1967). J. Mol. Biol. 29, 27.
13. Lodish, H. F. (1969). Nature, in the press.
14. Hershey, J. W. B., and Monro, R. (1966). J. Mol. Biol. 18, 68.
15. Anderson, J., Dahlberg, J., Bretscher, M., Revel, M., and Clark, B. (1967). Nature 216, 1072.
16. Haenni, A.-L., and Lucas-Lenard, J. (1968). Proc. Nat. Acad. Sci., U.S. 61, 1363.
17. Erbe, R. W., and Leder, P. (1968). Biochem. Biophys. Res. Comm. 31, 798.
18. Tanaka, M., Kinoshita, T., and Masukawa, M. (1968). Biochem. Biophys. Res. Comm. 30, 278.
19. Lodish, H. F. (1968). J. Mol. Biol. 32, 681.
20. Sugiyama, T., and Nakada, D. (1968). J. Mol. Biol. 31, 431.
21. Nathans, D., Oeschger, M., Polmar, C., and Eggen, K. (1969). J. Mol. Biol. 39, 279.
22. Sugiyama, T., Hebert, R., and Hartman, K. (1967). J. Mol. Biol. 25, 455.
23. Anderson, J., Bretscher, M., Clark, B., and Marcker, K. (1967). Nature 215, 490.
24. Ohtaka, Y., and Spiegelman, S. (1963). Science 142, 493.
25. Eggen, K., Oeschger, D., and Nathans, D. (1967). Biochem. Biophys. Res. Comm. 28, 587.
26. Engelhardt, D., Webster, R., and Zinder, N. (1967). J. Mol. Biol. 29, 45.
27. Capecchi, M., (1967). J. Mol. Biol. 30, 213.