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Control of Translation by the Conformation of Messenger RNA

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ABSTRACT **This study concerns the question of whether protein biosynthesis is controlled by the conformation of messenger RNA. When R17 bacteriophage RNA was first incubated in the presence of Mg^{++} before being used as messenger in a cell-free system from *Escherichia coli*, significant changes in the incorporation of phenylalanine and histidine into protein were seen. The amounts of proteins synthesized were also altered. The changes were not due to degradation of R17 RNA nor to denaturation of a contaminating repressor; they could be reversed by appropriate treatment of the RNA. Three forms of R17 RNA are therefore postulated for the control mechanism and characterized by their translational behavior. The significance of these forms with respect to their conformation is discussed.**

It has been shown that RNA from RNA-containing bacteriophages such as f2, MS2, and R17 have at least three cistrons —A, B, and C. It has been proposed as well that these three cistrons are translated sequentially, but not simultaneously, starting from B, namely, the coat protein cistron. This was supported by the observation (1–3) that in cell-free protein biosynthesis directed by phage RNA, the incorporation of histidine, which is absent in coat protein, was delayed by about 6 min with respect to that of lysine or valine, which are found in the coat protein.

However, in the course of our investigation of *in vitro* protein synthesis, some preparations of R17 RNA scarcely showed any delay in histidine incorporation. Similar results have been observed by Lodish (4). He further reported (5) that only the A cistron of f2 bacteriophage RNA is translated in a system containing *Bacillus stearothermophilus* 30S ribosomal subunits. On the other hand, Steitz *et al.* recently reported (6) that the efficiencies of ribosome binding to the initiation sites of the three genes are affected by initiation factors from T4 phage-infected *Escherichia coli*. Thus, it might be suggested that the translational order of the genes in R17 RNA is not determined by a linear sequence of cistrons, but by some other factors, including the conformation of the RNA molecule. In this communication we present results showing that the translational sequence does depend on the conformation of the RNA molecule.

MATERIALS AND METHODS

Cell-free extracts of *E. coli*

For the preparation of cell-free extracts, *E. coli* Q13 was grown in a medium containing, per liter: 5 g of polypeptone, 1 g of NH_4Cl, 8.8 g of $Na_2HPO_4 \cdot 12\ H_2O$, 1.5 g of KH_2PO_4, 10^{-5} mol of $MgSO_4$, 10^{-8} mol of $FeCl_3$, and 4 g of glucose. The cells were harvested when the density was reached 2×10^8 cells/ml and washed twice with TMK buffer (10 mM Tris·HOAc, pH 7.4–10 mM $Mg(OAc)_2$–50 mM KCl). The cell paste was ground with 2.5 g of quartz sand and extracted with 1.5 ml of TMK buffer per gram wet weight of cells. The extract was centrifuged at 12,000 $\times$ *g* for 15 min. The resulting supernatant fraction was made 6 mM in β-mercaptoethanol, and DNase (Worthington, electrophoretically pure) was added to a concentration of 5 μg/ml. It was centrifuged at 100,000 $\times$ *g* for 60 min. The upper four-fifths of the supernatant fraction was carefully taken up and dialyzed against TMK buffer containing 6 mM β-mercaptoethanol. This fraction was stored at −70°C in small aliquots until needed. Sucrose density gradient centrifugation of this fraction showed that native 50S and 30S ribosomal subunits were present to a much greater extent than 70S ribosomes. We ascertained that this extract incorporated scarcely any amino acids in the absence of R17 RNA.

Suppressor tRNA

Su I^+ suppressor tRNA, which inserts serine for the UAG codon, was prepared from permissive strain *E. coli* S26R1E (generously supplied by Dr. G. Gussin) in the manner described by Cappechi (7).

R17 RNA

R17 bacteriophage and Sus 3 amber mutant of f2 phage were generous gifts from Drs. D. G. Gussin and from K. Horiuchi (University of Tokyo), respectively. They were grown on *E. coli* A19 and *E. coli* S26R1E, respectively. Phage growth, purification, and phage RNA extraction were performed according to the procedure of Gesteland and Boedtker (8). For CsCl-banding purification, 7.95 g of CsCl was added to 13.0 ml of phage suspension and the whole was centrifuged in Spinco SW39 rotor at 36,000 rpm for 14 hr.

"Preincubated R17 RNA" refers to R17 RNA heated at a specified temperature in TM buffer (TMK buffer without KCl) for 3 min, then chilled rapidly (unless otherwise noted).

In vitro protein synthesis

Amino acid-incorporating experiments directed by R17 RNA were done with the *E. coli* extract described above. Components were: 30 μl of *E. coli* extract in 0.1 ml of standard reaction mixture; 0.03 mM amino acid (each one other than the ^{14}C- or ^{3}H-labeled one); 100 mM NH_4Cl; 10 mM $Mg(OAc)_2$; 25 mM Tris, final pH 7.4; 6 mM β-mercaptoethanol; and 30 μg of tRNA (purchased from General Biochemicals); 5 μg of creatine kinase; 4 μM [U-^{14}C]L-histidine (New En-

Abbreviation: TMK buffer, 10 mM Tris·HOAc (pH 7.4)–10 mM $Mg(OAc)_2$–50 mM KCl.

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gland Nuclear Corp., 250 Ci/mol); either 5 μM [G-^{3}H]L-phenylalanine (Radiochemical Centre, 750 Ci/mol) or 2 μM [4,5-^{3}H]L-leucine (Radiochemical Centre, 22.2 Ci/mol); 0.4 mM GTP; 3 mM ATP: 8 mM creatine phosphate; 3 μg of Ca-folinate; 30 μg of R17 RNA. Incubation was at 37°C; 50-μl aliquots were removed at intervals for determination (9) of ^{3}H and ^{14}C in material insoluble in hot 5% trichloroacetic acid (TCA).

Electrophoresis

A sample (100–200 μl of the reaction mixture) was incubated at 0°C, then 25°C, for (each) 15 min with added RNase IA and RNase T_1 (2 μg/ml of each). Free radioactive amino acids were removed on a Sephadex G-25 column, the effluent of which was applied to a gel. The 10% polyacrylamide gel (pH 7.2) was prepared and radioactivity was detected by the slicing method according to the procedure described by Nathans *et al.* (10). Electrophoresis was carried out at 5°C for about 3 hr at 12 mA/gel.

RESULTS

The results in Fig. 1 represent the time course of incorporation of both phenylalanine and histidine into hot 5% TCA-insoluble material synthesized in the *E. coli* extract, directed by R17 RNA. With unheated (0°C) RNA, amino acids other than histidine were incorporated after an intrinsic initial lag of about 1 min; the incorporation of histidine shows only about 1 min further delay. However, with RNA preincubated in TM buffer at various temperatures for 3 min, the incorporation profile changed significantly. RNA heated at 60°C showed a definite delay in histidine incorporation, while the RNA heated at 70°C did not show this delay. Furthermore, the incorporation rate of phenylalanine decreased when the preincubation temperature was 60°C, but increased abruptly when it was 70°C. The initial delay (1 min) did not change for phenylalanine incorporation.

These changes in incorporation profile cannot be due to the breakdown of R17 RNA since both heated and untreated RNAs showed a sharp single sedimentation peak (sedimentation coefficient 27 S). Similar results have been obtained by sucrose density gradient centrifugation or polyacrylamide gel electrophoresis of the RNAs.

A change of preincubation time, in the range from 3–15 min, did not bring about any significant difference in the

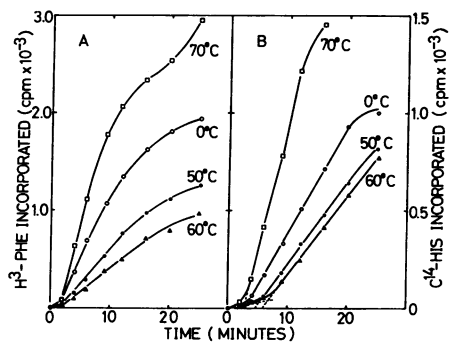


FIG. 1. Effect of preincubation temperature on the time course of incorporation of [^{3}H]phenylalanine (*A*) and [^{14}C]-histidine (*B*) into material insoluble in hot trichloroacetic acid. R17 RNA was incubated at 0°C (○), 50°C (●), 60°C (△), or 70°C (□) for 3 min in TM buffer, then chilled in an ice-bath. The reaction took place at 37°C.

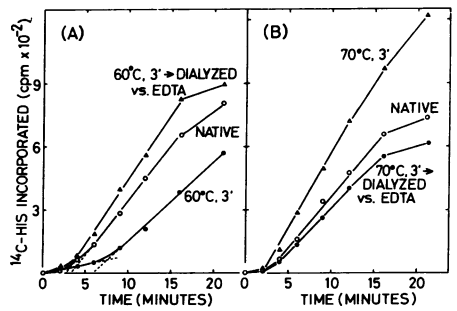


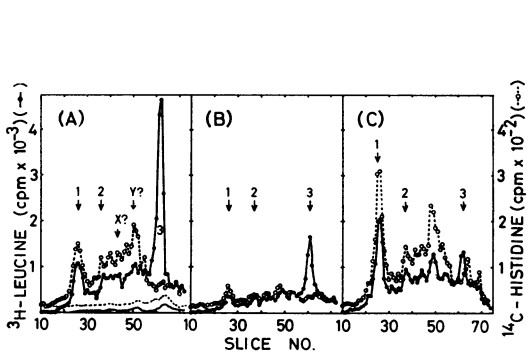
FIG. 2. Reversibility of histidine-incorporation profile. *A*, after preincubation of R17 RNA with 10 mM Mg^{++} at 60°C (increased lag in histidine incorporation, restored to normal by removing Mg^{++} by dialysis against EDTA followed by incubation at 45°C for 3 min); *B*, after preincubation of R17 RNA at 70°C (higher incorporation rate, restored to normal in the same manner as in *A*).

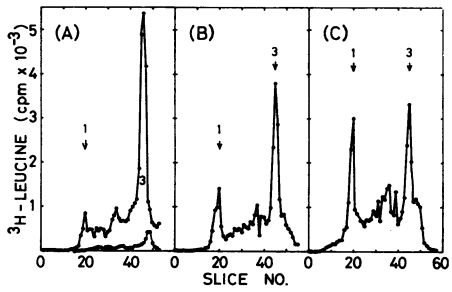
phenylalanine- or histidine-incorporation profiles characterized above. Nor was there a difference between RNA chilled rapidly and RNA annealed slowly from the preincubation temperature. Thus, it might be proposed that R17 RNA can take at least three forms. Form I corresponds to untreated RNA, which shows scarcely any delay in histidine incorporation. Form II can be derived from I by heating at 60°C in TM buffer. The incorporation rates of both histidine and phenylalanine are lower than those corresponding to form I, and the incorporation of histidine is delayed about 6 min when directed by the RNA of this form. Form III is obtained by heating RNA at 70°C in TM buffer. This form shows the highest incorporation rate of both phenylalanine and histidine and stimulates the incorporation of the latter without delay.

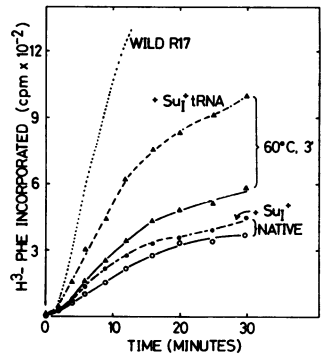
The presence of Mg^{++} during the pretreatment seems essential to attain either form II or form III, since heat treatment of R17 RNA in 10^{-3} M EDTA or in 0.15 M NaCl–0.015 M sodium citrate solution led to no change in the incorporation profile of histidine or phenylalanine. It should also be emphasized that the conversion between these forms is reversible. Form II could be reconverted to form I by mild incubation after dialysis against 10^{-3} M EDTA, as shown in Fig. 2*A*, which indicates the disappearance of the delay in the incorporation of histidine. The results of Fig. 2*B* prove that form III (with high incorporation rate) could be converted to form I by dialysis against 10^{-3} M EDTA.

These results clearly suggest that form II and III are not degraded products of form I, as has already been proved by sedimentation experiments. Furthermore, the possibility that heat denaturation of a repressor contaminating the R17 RNA preparation might change the translational behavior of the messenger can be excluded. In this respect it is interesting (14, 15) that some tRNAs can take two interchangeable forms mediated by Mg^{++}.

To clarify the translational differences observed for the three forms, we have examined the translational products by polyacrylamide gel electrophoresis. In Fig. 3, the translational products in a 13-min incubation had been labeled with [^{3}H]-leucine and [^{14}C]histidine. Peaks are denoted according to Eggen *et al.* (3, 11): peaks 1, 2, and 3 correspond to replicase, maturation protein, and coat protein, respectively. Peak 3 evidently corresponds to coat protein, since incorpora-







(*Left*) FIG. 3. Proteins synthesized in 13 min at 37°C, analyzed by polyacrylamide gel electrophoresis. Messengers: *A*, form I (native) RNA; *B*, form II (form I heated with Mg^{++} to 60°C); *C*, form III (heated to 70°C). The pattern with no added RNA is also shown in *A*. —●—, [^{3}H]leucine; --○--, [^{14}C]histidine. *1*, replicase protein; *2*, maturation protein; *3*, coat protein; *X*, *Y*, unknown.

(*Center*) FIG. 4. Proteins synthesized in 26 min at 37°C, analyzed by polyacrylamide gel electrophoresis. Labeled with [^{3}H]leucine. Otherwise as in Fig. 3.

(*Right*) FIG. 5. Effect of preincubation of Sus 3 RNA on its *in vitro* translation. The RNA from the Sus 3 strongly polar mutant was used as template in the cell-free amino acid-incorporating system, with or without suppressor tRNA (Su I tRNA). The Sus 3 RNA was pretreated as described below and suppressor tRNA was substituted for tRNA in the reaction mixture as follows: (○) native Sus 3 RNA, no Su I tRNA; (●) native Sus 3 RNA with Su I tRNA; (△) Sus 3 RNA preincubated (3 min) at 60°C in TM buffer, no Su I tRNA; (▲) Sus 3 RNA preincubated at 60°C in TM buffer, Su I tRNA in reaction mixture. The incorporation rate of phenylalanine directed by wild-type R17 is also indicated (——). Other experimental conditions as in Fig. 1.

tion of histidine cannot be observed for this peak. Peaks X and Y are unknown products; it has been suggested (3) that they represent precursors or degradation products of the above proteins or peptidyl-tRNA. The peak corresponding to maturation protein is not very clear. This might be due to degradation of this protein during RNase treatment since the protein is known (11) to be unstable on longer incubation. The differences of the three patterns are quite clear. Form I produces a lot of coat protein, as well as an ample amount of replicase. The product of form II is mostly coat protein. Although peak I could be observed slightly, this disappeared completely when the incubation time was shortened to 8 min. The products of form III are most striking. It is evident from the figure that the main product is replicase, while a little coat protein is synthesized.

When the translation was carried out for 26 min, the products of the three forms gave the patterns shown in Fig. 4. It can be seen that the accumulation of coat protein has already inhibited the synthesis of the other two proteins in the case of form I, since the ratio of peak 3 to peak 1 is far larger than that of Fig. 3. The products of form II are somewhat similar to those of form I, although the amounts of synthesized materials are smaller. Form III has synthesized the smallest amount of coat protein but the most replicase.

In summarizing Figs. 3 and 4, the following can be deduced. Form I starts the synthesis of all three proteins almost simultaneously. But, when some coat protein has accumulated, the synthesis of the other two proteins is inhibited. Translation of form II seems to proceed sequentially, beginning from coat protein. The rate of translation of this form is smaller than the other two, in accordance with the incorporation results above. Translation of form III starts from replicase. Coat protein, and probably maturation protein, are synthesized at a later stage.

To test this interpretation, we have used the RNA of Sus 3 amber mutant of f2 phage. Since this mutant RNA expresses strong polarity *in vitro* (2, 13), regular protein synthesis will not occur without the supplement of suppressor tRNA, so long as translation starts from the coat protein cistron and proceeds sequentially. In other words, the effect of suppressor tRNA should be marked only when translation proceeds sequentially from coat protein. From Fig. 5 it is evident that protein biosynthesis by Su I tRNA is stimulated only when form II of amber RNA is used as a template. Further experiments with Sus 3 RNA are now in progress.

DISCUSSION

One of the models that have been proposed to explain why translation of phage RNA proceeds sequentially (4, 12) assumes that translation starts from the 5′ terminus of phage RNA and proceeds along the RNA chain. Another model assumes that because of the specific conformation of phage RNA, only the initiation codon of the first cistron is exposed. As the translation of the first cistron proceeds, some conformational change exposes the initiation codons of other cistrons. These two models have a serious influence on explaining the polarity phenomenon observed for polycistronic amber-mutant RNA (12, 13).

We have to emphasize here that our three forms of phage RNA direct protein synthesis quite differently. Form I directs the synthesis of three proteins almost simultaneously: there is no delay in histidine incorporation, and the three proteins are present at any stage of synthesis. Form II directs the synthesis of coat protein first, and then replicase and maturation protein, probably sequentially; there is a distinct delay in histidine incorporation, and only coat protein appears as an early product of synthesis. The rate of synthesis is rather low. Form III directs preferentially the synthesis of replicase, which is the sole early product in synthesis directed by this form; consistent with this is the fact that no delay could be observed for histidine incorporation. Coat protein and probably maturation protein are synthesized later. However, it is difficult to discuss the sequence of synthesis precisely, since

the estimation of maturation protein proved difficult in our experiment.

From the profile of incorporation of phenylalanine and histidine, as well as from the analysis of translation products of the three forms, the following models can be suggested. Forms II and III have specific conformations that promote their translation sequentially. Form II starts its translation from coat protein, form III from replicase. Form I must have a conformation that facilitates the translation of two or three cistrons simultaneously. These models can explain not only the results cited here but also the apparently contradictory results reported for the translational sequence of phage RNA (3, 4) and for its mechanism (12, 13).

The results for form III are of particular interest. Although this form might be merely an experimental artifact, translation similar to that observed for form III may happen at the infection stage of RNA phage. Since replication of RNA would be preferentially necessitated for the production of daughter phage, it would be quite natural to assume that translation should start from replicase.

If it is assumed that the sequence of translation is mediated by conformation, the same idea is expected to be applicable to polarity phenomena. As shown above, Su I tRNA was effective in restoring incorporation only when form II of the mutant RNA was applied as template. Since Sus 3 amber mutant of f2 phage has a mutation in coat protein cistron, the addition of Su I tRNA should not be effective unless translation proceeds sequentially from coat protein. It is tempting to conclude that the observed polarity results from the translation not proceeding enough to unfold the specific conformation that makes the initiation codons of the second and third cistrons accessible.

Since the circular dichroic spectra of all three forms are alike, they cannot be very different in their secondary structures. They probably differ more in tertiary structure.

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1. Ohtaka, Y., and S. Spiegelman, *Science*, **142**, 493 (1963).
2. Engelhardt, D. L., R. E. Webster, and N. D. Zinder, *J. Mol. Biol.*, **29**, 45 (1967).
3. Eggen, K., and D. Nathans, *J. Mol. Biol.*, **39**, 293 (1969).
4. Lodish, H. F., *Nature*, **220**, 345 (1968).
5. Lodish, H. F., *Nature*, **226**, 705 (1970).
6. Steitz, J. A., S. K. Dube, and P. S. Rudland, *Nature*, **226**, 824 (1970).
7. Capecchi, M. R., *J. Mol. Biol.*, **21**, 173 (1966).
8. Gesteland, R. F., and H. Boedtker, *J. Mol. Biol.*, **8**, 496 (1964).
9. Mans, R. J., and G. D. Novelli, *Arch. Biochem. Biophys.*, **94**, 48 (1961).
10. Nathans, D., M. P. Oeschger, K. Eggen, and Y. Shimura, *Proc. Nat. Acad. Sci. USA*, **56**, 1844 (1966).
11. Eggen, K., M. P. Oeschger, and D. Nathans, *Biochem. Biophys. Res. Commun.*, **28**, 587 (1967).
12. Zinder, N. D., D. L. Engelhardt, and R. E. Webster, *Cold Spring Harbor Symp. Quant. Biol.*, **31**, 251 (1966).
13. Lodish, H. F., *J. Mol. Biol.*, **32**, 681 (1968).
14. Ishida, T., and N. Sueoka, *Proc. Nat. Acad. Sci. USA*, **58**, 1080 (1967).
15. Adams, A., T. Lindahl, and J. R. Fresco, *Proc. Nat. Acad. Sci. USA*, **57**, 1685 (1967).