DYNAMICS OF DNA-DEPENDENT RNA SYNTHESIS IN A CELL-FREE SYSTEM OF Escherichia coli

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The reaction of RNA synthesis in a cell-free system of Escherichia coli stops after 15 min. Addition every 15 min of a supernatant obtained by centrifuging destroyed bacterial cells and containing RNA-polymerase and endogenous DNA template restarts the reaction of RNA synthesis in the presence of the original content of nucleoside triphosphates. If supernatant is added periodically, far more RNA is synthesized than if the corresponding amount of triphosphates is added.

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The synthesis of RNA in vitro on a DNA template has attracted the attention of many investigators. The work has been carried out principally in two directions: to obtain purified preparations of RNA-polymerase and to study the mechanisms of transmission of genetic information from DNA to RNA.

Synthesis of RNA by means of RNA-polymerase on native DNA proceeds initially with linear velocity, after which the curve reflecting the dynamics of synthesis becomes a plateau, i.e., the reaction practically ceases [3-6, 10]. This course of the curve is characteristic of all stages of purification of RNA-polymerase [8].

In 1962 the hypothesis was put forward [9] that the reaction stopped because of a fall in the concentration of ribonucleoside triphosphates. By adding fresh portions of triphosphates every 10 min, continuous synthesis for 60 min was achieved, and in this way the usual curve of the RNA-polymerase reaction was modified. However, the way in which addition of other components to the system influences the course of the reaction remained unexplained.

In the investigation to be described an attempt was made to prolong the reaction of RNA synthesis by adding supernatant, keeping the content of triphosphates unchanged, and studying the dynamics of the RNA-polymerase reaction in these conditions.*

To study these problems we used the system for DNA-dependent RNA synthesis proposed and described by Otaka and co-workers [9].

EXPERIMENTAL METHOD

Growth of the culture of E. coli B is described elsewhere [2].

Preparation of RNA-polymerase and Determination of Its Activity. Cells of E. coli in the logarithmic phase of growth were washed twice with a solution containing 0.025 M Tris-HCl buffer, pH 7.5, 0.01 M Mg $(CH_3COO)_2 \cdot 4H_2O$, 0.001 M $MnCl_2 \cdot 4H_2O$, and 0.05 M NH_4Cl, ground with glass sand, extracted with three times (relative to fresh weight of the microorganisms) the amount of the same solution. The mixture was centrifuged at 3800 g for 30 min and again for 2 h at 105,000 g. The supernatant obtained at 105,000 g (1 ml supernatant contained about 2 mg protein, 0.2 mg DNA, and 0.4-0.5 mg RNA) was used as RNA-polymerase preparation on the day it was obtained. All operations were carried out at 0-4°.

The system for testing RNA-polymerase activity consisted of 5 μmoles $Mg(CH_3CO)_2 \cdot 4H_2O$, 1 μmole $MnCl_2 \cdot 4H_2O$, 25 μmoles NH_4Cl, 12.5 μmoles Tris-HCl buffer, pH 7.5, 0.25 μmole each of GTP, CTP, and

*Supernatant obtained by centrifuging disintegrated bacterial cells for 2 h at 105 000 g, containing endogenous DNA template and unpurified RNA-polymerase. This is what is meant by supernatant throughout the text.

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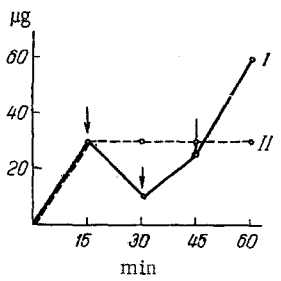

Fig. 1. Dynamics of RNA synthesis on addition of supernatant every 15 min (I) and leaving the initial amount of supernatant in the system (II). Incubation at 37°. RNA content determined from optical density of alkaline hydrolysate.

TABLE 1. Amount of RNA (in μg) Synthesized during Incubation for 60 min with Addition of Triphosphates or Supernatant

Expt. No.	In first 15 min	In 60 min without additions	In 60 min with addition, every 15 min, of	
			triphosphates	supernatant
1	10	–	20	38
2	–	10	20	40

UTP, 1 μmole ATP, 20 μg pyruvate kinase, 2.5 μmole phosphoenolpyruvate, 15 μg protamine sulfate, and 0.2 ml supernatant. The final volume was 0.5 ml. Incubation took place at 37°.

At the end of incubation the reaction was stopped with cold 3N $HClO_4$ (final concentration 0.5 N), the residue of nucleic acids was washed 3 times with 0.5N $KClO_4$ (in portions of 5 ml) with centrifugation in the cold for 20 min (15,000 g) to remove acid-soluble products. The acid-insoluble residue, containing the two nucleic acids, was subjected to alkaline hydrolysis (0.5 ml 1N KOH, 18 h, 37°), after which the DNA was precipitated with cold 3N $HClO_4$ solution (0.2 ml) and the RNA contained in the supernatant fluid was collected by washing the DNA residue three times with 0.2 N $HClO_4$ pooled and made up to the same volume.

The RNA concentration was determined by measuring absorption on a type SF-4A spectrophotometer, using the formula [1]:

$$\frac{E_{270} - E_{290}}{0.19} \cdot 10.5 \cdot \text{dilution} = x \ \mu g \text{ RNA/ml},$$

where E represents the extinction at that wavelength. Unincubated samples containing the same components as the experimental, and treated similarly, were used as controls.

In the experiments in which supernatant was added at 15 min intervals, a control was set up for each point ("zero time"), when an amount of supernatant equal to the total amount added to the experimental samples was introduced at once.

The results of the parallel tests were in good agreement. The maximal discrepancy between the results of these samples by the spectrophotometric method was 3 μg and 20 pulses/min in the incorporation experiments.

Determination of RNA by Incorporation of C^{14}-uridine Monophosphate (C^{14}-UMP; 0.16 μCi, Specific Activity 0.76 μCi/g). In this case the sytem contained 1 labeled triphosphate and 3 unlabeled, but otherwise the composition of the samples was the same as described above. Incubation and treatment of the samples were carried out as by the spectrophotometric method (see above), but after removal of the acid-soluble material they were washed once more with a mixture of ethanol and ether (1:1), dissolved in formic acid, transferred quantitatively to targets, dried, and their radioactivity was determined in a gas-flow counter.

EXPERIMENTAL RESULTS

In the first experiments the effect of periodic addition of triphosphates and supernatant containing template and RNA-polymerase on the amount of RNA synthesized was compared. Two series of incubation mixtures were used. To one series an equal amount of all four triphosphates to that present initially was added every 15 min during incubation for 1h, while to the other series 0.2 ml of supernatant was added.

TABLE 2. Amount of RNA (in μg) in Samples during Incubations with Supernatant for 60 min

Expt. No.	Type of RNA	Initial RNA content in sample	Total RNA content (exogenous + background) before incubation	Total RNA content after incubation	RNA broken down during incubation
1	Exogenous	20	134	116	18
2		20	298	278	20
1	Endogenous	273	–	273	0
2	(background)	233	–	233	0

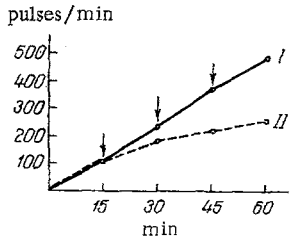

Fig. 2. Dynamics of incorporation of C^{14}-uridine monophosphate (from uridine triphosphate) into acid-insoluble fraction on addition of supernatant every 15 min (I) and when initial content of supernatant present only (II).

The results of these experiments are presented in Table 1. They show that addition of the matrix and enzyme (i.e., or supernatant containg them) was considerably more effective than the addition of triphosphates. Stimulation of RNA synthesis by addition of supernatant led to the synthesis of appreciable amounts of RNA in vitro and to the more complete utilization of the added precursors.

The dynamics of RNA synthesis with and without addition of supernatant was then studied. The experiments were performed just as in the preceding investigations, but every 15 min during incubation the RNA content was determined in parallel samples. Figure 1, which illustrates typical results, shows that curve II has the usual appearance for an RNA-polymerase reaction and demonstrates that the increase in RNA stopped in the first 15 min in the absence of additions. Curve I, reflecting the dynamics of RNA synthesis on addition of supernatant to the system, is of a completely unusual shape.

The experiments were repeated several (more than 10) times, and the shape of the curve was always well reproduced. A series of control experiments was set up to exclude any possible influence of accidental causes, such as the pH and temperature of the added supernatant.

In an attempt to explain the unusual character of curve I, we postulated that it is the "resultant" of the curves of RNA synthesis and breakdown. However, as can be seen in Fig. 1 (curve II), after incubation for 15 min the amount of RNA in the system was stabilized. This could have occurred for two reasons: either all processes of RNA synthesis and breakdown were severely retarded, or they still took place but compensated each other accurately. Addition of supernatant after 15 min caused the breakdown of part of the RNA, while subsequent portions stimulated synthesis sharply.

It is interesting to note that the RNA present in the supernatant (background) was unchanged in quantity during incubation for 60 min. This was shown by the results of experiments in which the reaction mixture was incubated in the absence of one of the four triphosphates, i.e., in conditions ruling out the possibility of RNA synthesis. Hence, curve I evidently reflects only the changes affecting newly synthesized RNA.

Meanwhile the exogenous RNA added to the system was readily broken down during incubation for 60 min under the influence of the nucleases contained in the supernatant (Table 2). Probably the background RNA present in the supernatant is resistant to nuclease action. It may perhaps be soluble RNA or RNA forming a complex with DNA; both these forms are known to be resistant to ribonuclease action.

In all the experiments described above the RNA content was determined spectrophotometrically. To verify the results obtained by the use of a more sensitive method, experiments were carried out in which RNA synthesis was estimated from incorporation of C^{14}-UMP.

It will be clear from Fig. 2 that, without addition of supernatant, the intensity of incorporation fell slowly (curve II) during incubation for 60 min. On addition of supernatant every 15 min, incorporation took place with linear velocity throughout the period of incubation, and breakdown was never once observed. If breakdown of the synthetized RNA had actually occurred, it must have been detected by this more sensitive method.

Hence, curve I in Fig. 1 (RNA determined spectrophotometrically) demonstrates that between the 15th and 30th min of incubation part of the RNA was broken down. Analysis of curve I in Fig. 2 (RNA determined from incorporation of C^{14}-UMP), on the other hand, demonstrates that no breakdown of radioactive material took place.

Comparison of the spectrophotometric and radioactive incorporation methods of determining RNA revealed two differences between them. The spectrophotometric method determined not only the newly synthesized RNA, but also the background RNA present in the supernatant. Admittedly as stated above, in the experimental conditions used it was not broken down in the course of 60 min. RNA breakdown observed by use of the spectrophotometric method cannot therefore be explained by breakdown of the background RNA. The spectrophotometric method of RNA assay included a stage of alkaline hydrolysis not present in the radioactive labeling method. Possibly the RNA breakdown observed by the spectrophotometric method may be attributed to the appearance of an RNA fraction in our system resistant to alkaline hydrolysis, which could give visible evidence of breakdown of part of the synthesized RNA. So far as we know, an RNA of this type, resistant to alkaline hydrolysis, is found in DNA from the liver mitochondria of adult rats [7].

The mechanism of this unusual action of added supernatant on the processes of RNA synthesis remains unclear. Further investigations are being undertaken in order to shed light on this problem.

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