

Coupling of Replication to Transcription *In Vitro*

(RNA polymerase/DNA polymerase I/bacteriophage f1 DNA/synergistic effect)

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Communicated by Erwin Chargaff, June 13, 1972

ABSTRACT In a coupled system consisting of RNA polymerase and DNA polymerase I of *Escherichia coli*, the four deoxyribo- and the four ribonucleoside triphosphates, and DNA of bacteriophage f1 as template, DNA synthesis depends on the concomitant synthesis of RNA. Over a wide range of concentrations of the two polymerases, RNA synthesis was unaffected by the simultaneous synthesis of DNA, whereas the rate of DNA synthesis depended on the level of RNA synthesis. In the coupled reaction, RNA synthesis starts immediately at a high rate, which subsequently decreases, whereas DNA synthesis starts after a lag and its rate increases as the reaction proceeds. Upon addition of rifampicin, the rate of RNA synthesis falls abruptly, while that of DNA declines only gradually. The base composition of the DNA synthesized in the coupled reaction is complementary to that of f1 DNA template. It is suggested that the RNA synthesized by the RNA polymerase serves as a primer rather than as a template for the DNA polymerase.

A recent communication from this laboratory (1) describing a DNA polymerase from chicken embryo preferring a DNA-RNA hybrid template included the observation that DNA synthesis by that enzyme could be stimulated by the concomitant transcription of the same template with RNA polymerase. This first demonstration of an *in vitro* synergism between the two types of polymerases was followed by reports from other laboratories, suggesting a similar situation *in vivo* (2-6). We have subsequently extended this observation by demonstrating a cooperative effect of the RNA and DNA polymerases of *E. coli* acting on isolated single-strand fractions of *Bacillus megaterium* DNA (7).

The present report consists of a more detailed study of this *in vitro* coupling of replication to transcription, using *E. coli* RNA polymerase, *E. coli* DNA polymerase I, and, as a template, bacteriophage f1 DNA. The effects of changes in the relative concentrations of the two enzymes are examined, as well as the kinetics of the reaction in the presence or absence of rifampicin. An analysis of the base composition of the synthesized DNA is presented.

MATERIALS AND METHODS

Enzymes. DNA polymerase I (EC 2.7.7.7) was prepared from *Escherichia coli* according to published procedures (8, 9). The preparation had a specific activity (defined and determined as in ref. 9) of 15,600 units/mg of protein. RNA polymerase (EC 2.7.7.6) was isolated from *E. coli* by a modification (J. G. Stavrianopoulos, unpublished) of a recent procedure (10); when assayed with native calf-thymus DNA (10 min, 37°), it promoted the incorporation of 825 nmol of CTP per mg of protein.

Template DNA. The strains of bacteriophage f1 and *E. coli* K79 used were kindly provided by Drs. N. D. Zinder and P. Model. The *E. coli* host cells were grown in a medium containing 10 g of Bacto-tryptone, 2 g of yeast extract, 2 g of glucose, and 9 g of NaCl per liter and were infected when they reached a density of 5×10^8 cells per ml with one f1 phage per cell. After 5 hr, the cells were removed by low-speed centrifugation, and the phage was precipitated from the supernatant by the addition of polyethylene glycol (Carbowax 6000) to 5% and NaCl to 0.5 M (11). The phage was collected and purified by repeated cycles of low- and high-speed centrifugations in borate buffer (50 mM borate-5 mM EDTA, pH 9). f1 DNA was isolated from the purified phage by two phenol extractions, followed by prolonged dialysis to remove phenol (12). The base composition of this DNA was determined by perchloric acid hydrolysis and paper chromatography (13).

Precursors. Deoxyribo- and ribonucleoside triphosphates were purchased from Schwarz-Mann and Sigma. ³H-Labeled deoxyribonucleotides and [¹⁴C]ATP were products of Schwarz-Mann and New England Nuclear.

Assay Conditions. The standard assay mixture contained, in a final volume of 0.13 ml, 0.05 M Tris-HCl (pH 8.3); 1 mM MnCl₂; 0.05 M KCl; 20 nmol (each) of the four deoxyribonucleoside triphosphates with [³H]dTTP (25 cpm/pmol); 60 nmol (each) of the four ribonucleoside triphosphates with [¹⁴C]ATP (2000 cpm/nmol); 3.3 μg of f1 DNA; 0.75-24 μg of RNA polymerase; and 30-600 ng of DNA polymerase I. The reaction was started by the addition of the RNA polymerase, followed 15 sec later by the DNA polymerase. Incubation was at 37° for 90 min. The reaction was terminated by the addition of 1.5 ml of 10% trichloroacetic acid; after 15 min at 0°, the precipitates were collected on membrane filters (Schleicher & Schuell, type B-6), washed seven times with cold 5% Cl₃CCOOH, and counted in a dioxane-naphthalene scintillation mixture, in a Beckman scintillation counter.

RESULTS

Requirements of the coupled reaction

The requirements of the coupled transcription-replication reaction are shown in Table 1. Two sets of results are presented, with two different levels of the enzymes. The results are qualitatively identical. RNA polymerase transcribes f1 DNA almost equally well in the presence as in the absence of DNA polymerase or deoxyribo precursors. The requirements for RNA synthesis seem to be unaffected by the presence of

the DNA synthesizing system: template and Mn^{2+} are required, KCl stimulates synthesis, and rifampicin inhibits it—completely if first incubated with the RNA polymerase, and partially if added after the beginning of incubation.

DNA polymerase, on the contrary, can use $\phi 1$ DNA as template very poorly in the absence of RNA polymerase or RNA precursors. DNA synthesis, however, is stimulated almost 30-fold (20-fold with low enzyme concentrations) by the concomitant synthesis of RNA. DNA template is required for the reaction, as well as Mn^{2+} . KCl stimulates the reaction 2-fold with low concentrations of the enzymes, but much less with high concentrations. Rifampicin inhibits DNA synthesis almost completely if first incubated with the RNA polymerase; if added 5 min after the beginning of the incubation, it inhibits entirely DNA synthesis with low levels of enzymes, but has little effect when the enzymes are present at high concentrations.

Effect of variations in the relative concentrations of the two polymerases

The effects of variations in the relative concentrations of the two polymerases on the amounts of RNA and DNA synthesized in the coupled reaction are presented in Figs. 1 and 2. Fig. 1 shows the amounts of DNA and RNA synthesized with increasing concentrations of RNA polymerase, at two different levels of DNA polymerase. At concentrations of RNA polymerase above 1 μg per 0.13-ml assay, the amount of RNA synthesized increases linearly with the concentration of RNA polymerase. DNA synthesis also increases linearly

TABLE 1. Requirements of the coupled transcription-replication reaction

Conditions	Exp. 1*		Exp. 2†	
	Incorporation (pmol)			
	[3H]-dTMP	[^{14}C]-AMP	[3H]-dTMP	[^{14}C]-AMP
Complete system	4174	3046	1225	475
Omit DNA polymerase	36	3763	10	544
Omit RNA polymerase	150	19	68	18
Omit Ribo precursors	205	0	76	0
Omit Deoxyribo precursors	0	4032	0	534
Omit $\phi 1$ DNA	0	23	4	22
Omit Mn^{2+}	3	36	1	15
Omit KCl	3708	2688	605	165
Add Rifampicin (after 5 min)	3806	775	2	180
Add Rifampicin (5 min prior incubation)	444	87	77	17

The standard incubation mixtures and conditions were used, with the omission of the indicated components. Rifampicin (final concentration 20 $\mu g/ml$) was either added 5 min after the beginning of the incubation, or first incubated with the RNA polymerase for 5 min before the addition of the latter to the incubation mixture.

* 12 μg of RNA polymerase, 0.3 μg of DNA polymerase.

† 3 μg of RNA polymerase, 0.12 μg of DNA polymerase.

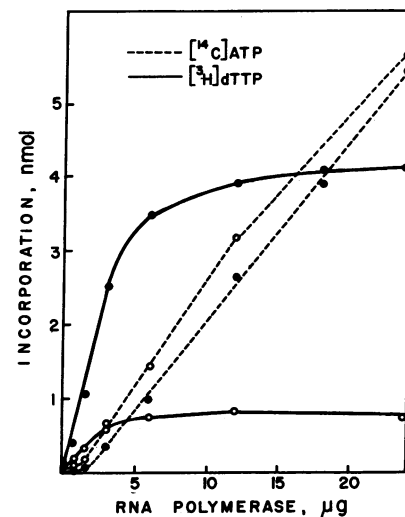


FIG. 1. Effect of variations in the concentration of RNA polymerase. The standard assay mixtures and conditions were used with the indicated amounts of RNA polymerase and either 60 ng (O) or 300 ng (●) of DNA polymerase.

with increasing concentrations of RNA polymerase, and reaches a plateau dependent on the amount of DNA polymerase present: the amount of DNA at the plateau reached with 300 ng of DNA polymerase is five times higher than that obtained with 60 ng of this enzyme.

Fig. 2 presents the results of the reverse experiment, i.e., increasing the concentration of the DNA polymerase while keeping that of the RNA polymerase constant; two different levels of RNA polymerase are used as well as a control without this enzyme. At both levels of RNA polymerase, RNA synthesis remains constant over the whole

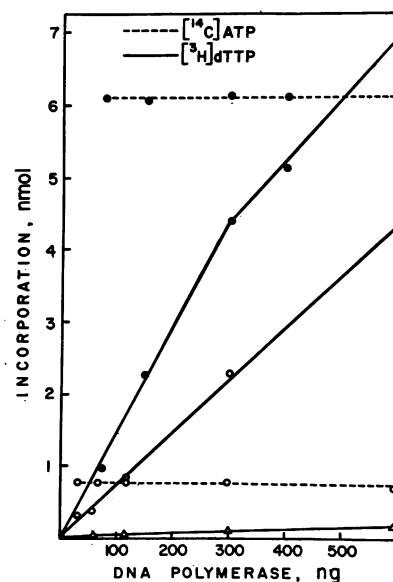


FIG. 2. Effect of variations in the concentration of DNA polymerase. The standard assay mixtures and conditions were used with the indicated amounts of DNA polymerase and no RNA polymerase (Δ); 3 μg of RNA polymerase (O), or 24 μg of RNA polymerase (●).

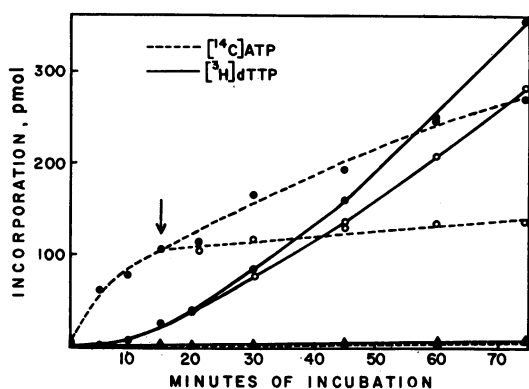


Fig. 3. Kinetics of the coupled reaction. A 15-fold standard assay mixture was prepared (final volume, 1.95 ml) and incubation was started by the addition of 45 μ g of RNA polymerase and 450 ng of DNA polymerase; 100- μ l samples were removed into tubes containing 1.5 ml of 10% Cl_2CCOOH at 0, 5, 10, and 15 min. At 15 min (arrow), half of the remaining reaction mixture was transferred into a tube containing 30 μ l of rifampicin solution (500 μ g/ml). At the times indicated, 100- μ l samples were removed from each tube into 1.5 ml of Cl_2CCOOH . (●) without rifampicin; (○) with rifampicin added at 15 min. The curves with the RNA polymerase first incubated with rifampicin (▲) were obtained in a separate similar experiment.

range of concentrations of DNA polymerase. The amount of DNA synthesized increases linearly with increasing concentration of DNA polymerase, the slope of the curve depending on the level of RNA polymerase present. It is probably significant that a change in the slope of the curve with 24 μ g of RNA polymerase is observed at the point where the amount of DNA synthesized is about equal to the amount of DNA template present.

A comparison of Figs. 1 and 2 illustrates the contrasting behavior of the two enzymes: RNA polymerase action is essentially unaffected by the concomitant synthesis of DNA, whereas the level of DNA synthesis depends not only on the concentration of the DNA polymerase, but also on that of the RNA polymerase.

Kinetics of the reaction

The sequence of events in the coupled reaction was investigated by measurement of the rate of synthesis of both RNA and DNA, in the absence or presence of rifampicin. The results are presented in Fig. 3. Several features of the reaction can be seen in this figure. (a) RNA synthesis precedes that of DNA. (b) The rate of RNA synthesis is maximal during the first few minutes of the reaction, and decreases gradually thereafter, whereas that of DNA synthesis increases slowly in the beginning and becomes linear after 30–35 min. (c) If the RNA polymerase is first incubated with rifampicin, the synthesis of both DNA and RNA is practically abolished. (d) If rifampicin is added 15 min after the beginning of incubation, the rate of RNA synthesis falls abruptly; the new rate, presumably due to the elongation of chains initiated before the addition of the inhibitor, is about 15–20% of the initial. The rate of DNA synthesis, however, does not exhibit any sudden change, but rather a gradual slow decline; its divergence from the rate recorded in the absence of rifampicin becomes significant only after another 25–30 min have elapsed.

Composition of the product DNA

The base composition of the DNA synthesized in the coupled reaction was determined by a technique analogous to "transcription analysis," which was found to give remarkably accurate results (14, 15). The details are given in the legend of Table 2, which presents the results of this analysis. Table 2 also shows the base composition, determined by direct chemical analysis, of the f1 DNA used as template; included for comparison are the compositions of three other filamentous coliphages, M13, fd, and Φ X174, taken from the literature.

As seen in Table 2, the composition of the DNA synthesized in the coupled reaction appears to be complementary to that of the phage DNA used as template, which is very similar to the composition of the other filamentous viruses.

DISCUSSION

Experiments on the simultaneous action of RNA and DNA polymerases *in vitro* have been reported before. Hurwitz *et al.* (19) using the two polymerases, the four deoxyribo- and the four ribonucleotide precursors, and calf-thymus, T2 phage, or *B. subtilis* DNA as templates, came to the conclusion that each enzyme acted independently of the other. Berg *et al.*, on the other hand (20), reported that at low template concentrations, RNA polymerase inhibited DNA polymerase action. The failure to observe a synergistic effect on these occasions was most probably due to the fact that the DNA preparations used had by themselves enough primer sites for the DNA polymerase and no cooperation of the RNA polymerase was necessary.

We obtained the first positive results of a synergistic effect with a preparation of a chicken-embryo DNA polymerase and *E. coli* RNA polymerase acting simultaneously on denatured calf-thymus DNA (1). This observation was subsequently extended to a combination of RNA and DNA polymerases, both from *E. coli*, using as templates the isolated single-strand fractions of *B. megaterium* DNA. The single strands had no template activity for DNA polymerase, but

TABLE 2. Base composition of DNA synthesized in the coupled reaction and of f1 DNA used as template

Source of DNA	Base composition (mol %)				Reference
	A	G	C	T	
f1 (synthesized)	32.4	20.7	21.2	25.6	This work
f1 (template)	24.5	21.1	21.3	33.1	This work
M13	23.3	21.1	19.8	35.8	(16)
fd	24.4	19.9	21.7	34.1	(17)
Φ X174	24.6	24.1	18.5	32.7	(18)

Four identical standard assay mixtures were used, with 3 μ g of RNA polymerase and 30 ng of DNA polymerase. The ribonucleoside triphosphates were all unlabeled, and a different deoxyribonucleoside triphosphate was labeled in each of the four mixtures. Both the unlabeled and the labeled deoxyribo precursors used in this experiment were purified by chromatography on Dowex 1. The specific activity of each labeled precursor was accurately estimated by triplicate determinations of its absorbance and radioactivity. From the incorporations of the individual bases, the composition of the product DNA was computed. The composition of the template DNA was determined as mentioned in the text. The figures reported for both product and template DNA are the averages of two separate experiments, each performed in triplicate.

were converted to efficient templates after transcription with RNA polymerase and reisolated of the hybrids. The simultaneous action of the two polymerases, although quite efficient with synthetic poly(dT) as template, gave positive, but quantitatively poor, results with the *B. megaterium* DNA strands. In this case, a staggered reaction, involving first incubation with RNA polymerase alone, and a second step with DNA polymerase, was more successful (7).

In the present work with f1 DNA, it has been possible to achieve very high rates of DNA synthesis in the coupled reaction, and a clearcut 30-fold stimulation over the rate of synthesis with DNA polymerase alone. This synergistic effect was observed over a large range of concentrations of the two enzymes.

The small, but measurable, synthesis of DNA observed with DNA polymerase alone (about 3% of that in the coupled reaction) can most probably be explained by imperfections of the template preparation.

The experiments described above provide also answers to several questions with potential biological significance—if one assumes a parallel course of events *in vivo*. First of all, the synthesis of RNA and that of DNA do not begin simultaneously, and do not proceed at the same rate. As seen in Fig. 3, there is a lag in the appearance of DNA; the accumulation of a certain amount of RNA seems to be required before a significant rate of DNA synthesis can be reached. This could be due to the fact that DNA polymerase requires primer sites with free 3'-OH groups; while an RNA chain is being synthesized, its 3' end is occupied by the RNA polymerase; only those RNA molecules that have been released by the RNA polymerase—while remaining still hybridized to the DNA—can serve as primers for DNA synthesis. The observed time lag would, then, represent the time necessary for the completion of such RNA molecules. This explanation is corroborated by the rate of synthesis observed after addition of rifampicin (Fig. 3): although no new RNA chains are initiated, those already under way continue to be elongated, and eventually the RNA polymerase leaves their 3' ends; thus, the lack of primer sites for the DNA polymerase is not immediately apparent, its effects becoming significant only as the reaction proceeds.

An important question left unanswered by the previous work that can be answered now is whether the DNA synthesized in the coupled reaction is complementary to the single-stranded DNA added to the reaction mixture, or identical to it and complementary to the RNA strand produced during the reaction. Indeed, in the coupled reaction with poly(dT) as template reported before (Table 2 of ref. 7), the synergistic action of the two polymerases had resulted in the synthesis of poly(rA) and poly(dT), suggesting the possibility of a "reverse transcription" type of reaction, with the newly formed ribo strand acting as the template for the DNA polymerase. The results presented here (Table 2), on the contrary, indicate that in this case the composition of the DNA product is complementary to that of the DNA template. This result would seem to suggest again a primer function for the RNA, in agreement with the findings on the rate of the

reaction discussed above. It should be emphasized, however, that this answer cannot be automatically extended to other systems, since, as mentioned before, a synergism between the two polymerases resulting in "reverse transcription" has also been observed.

There are, of course, several interesting questions still to be answered. What are the lengths of the RNA and DNA chains synthesized, especially in view of the fact that in the course of the reaction the rate of DNA synthesis eventually exceeds that of RNA (Fig. 3)? Are short RNA oligonucleotides released, or only full-length copies of the entire f1 DNA molecule? Is the DNA product completely hybridized to the DNA template, and could this double-stranded DNA serve, in turn, as template for further synthesis of DNA identical to the original single strand? Can the replicative form of f1 DNA be used in the *in vitro* coupled reaction? With the information now at hand on some of the essential features of the reaction, it is possible to design experiments that could provide some answers to these questions.

I thank Dr. Erwin Chargaff for his support and encouragement, and Drs. N. D. Zinder and P. Model for providing the strains of phage f1 and *E. coli* K79. The excellent technical assistance of Mr. Richard Liou is gratefully acknowledged. This work was supported by Research Grant CA-12210-12 from the National Institutes of Health U.S. Public Health Service, and Grant NP-56N from the American Cancer Society.

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