TRANSCRIPTION ON AN RNA POLYMERASE - DNA COMPLEX FROM E. COLI: EVIDENCE FOR IN VITRO SYNTHESIS OF RIBOSOMAL RNA*

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As the major RNA component of the cell, ribosomal RNA[1] must undergo an extensive synthesis during the cell generation cycle. Indeed, the available experimental evidence indicates that the production of stable RNA components (which are primarily r-RNA) account for 20 to 70% of the total transcription in bacteria, depending on the bacteria and on the method of measurement (for examples see: Bolton and McCarthy, 1962; Leive, 1965; Mangiarotti and Schlessinger, 1967; Salser, Janin and Levinthal, 1968; Kennell, 1968.) The regulatory interactions which lead to this preferential synthesis of r-RNA are unknown. As a means for examining the phenomenon, a cell-free system which exhibits r-RNA synthesis would be useful.

In this report it is demonstrated that approximately 20% of the radioactivity in RNA synthesized from an RNA polymerase-DNA complex isolated from E. coli is indistinguishable from r-RNA in competition hybridization tests. By contrast RNA made in vitro from denatured E. coli DNA exhibits no detectable competition with r-RNA. The effects of heparin on the RNA polymerase-DNA complex suggest that most of the RNA synthesis occurs as an extension of nascent RNA chains isolated with the complex.

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[1]Abbreviations used: r-RNA, ribosomal RNA; d-DNA, denatured DNA; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0.

METHODS AND MATERIALS

The RNA polymerase-DNA complex was prepared from lysozyme lysates of E. coli strains B or MRE 600 (RNase I$^-$) by means of multiple extractions in the polyethyleneglycol-dextran two-phase system. The procedure used was as previously described (Favre and Pettijohn, 1967) except that only the first three extractions of this sequence were utilized. These steps were sufficient to eliminate the free high molecular weight RNA from the preparation and to provide a partially purified protein-DNA complex which contained 60 to 80% of the DNA and approximately 70% of the RNA polymerase activity present in the original cell-free lysate.

The reaction mixture for RNA synthesis contained in a volume of 0.25 to 1.0 ml: 0.033 M tris (pH 7.8); 10 mM $MgCl_2$; 0.10 M KCl; 0.20 mM each of ATP, GTP and UTP; 0.058 mM H^3 labeled CTP - 1700 mC/m-mole; and approximately 100 µg/ml DNA equivalent of the partially purified protein-DNA complex. After 4 min at 37°, the reaction was terminated by the addition of SDS to a final concentration of 1.0%. The labeled RNA was then purified by phenol and chloroform-octanol extractions with recovery steps, ethanol precipitations, DNase treatment (Worthington RNase free), followed by more phenol and chloroform-octanol extractions, ethanol precipitation, resuspension in 6 X SSC, a heat treatment to inactivate any remnant DNase, and filtration through a Schleicher and Schuell B-6 nitrocellulose filter. The specific activity of the purified H^3-RNA was 15,000 to 33,000 cpm/µg and 120% to 200% of the initial trichloracetic acid precipitable counts were recovered (the increase probably results from the elimination of contaminants which absorb and quench the weak β particles.)

The synthesis and purification of H^3-RNA from E. coli d-DNA template followed the same procedure except that H^3-CTP at 1000 mC/m-mole was used and purified E. coli DNA which had been heated (90° for 5 min in 5 mM tris) and purified E. coli RNA polymerase were substituted in the reaction mixture in place of the complex at concentrations of 70 µg/ml and 58 µg/ml respec-

tively. Prior to DNase treatment the RNA-DNA hybrids were disassociated by heating. The specific activity of this RNA was 120,000 cpm/μg.

Radioactive r-RNA was prepared by the method of Yankofsky and Spiegelman (1963) from E. coli cells which had been grown with H^3-uracil and then chased with non-radioactive uracil for several generations. The specific activity was 22,000 cpm/μg. Non-radioactive r-RNA was extracted with SDS and phenol from separate preparations of purified 30 and 50S ribosomes from E. coli strain B. These two r-RNA fractions were then further purified by sedimentation through sucrose gradients containing 0.5% SDS, 0.10 M NaCl, 0.01 M tris 8.1.

Hybridization methods were those of Gillespie and Spiegelman (1965). The E. coli DNA used in this technique was isolated by a method previously described (Favre and Pettijohn, 1967) or purchased (Worthington). DNA from phages T_4 and T_7 was prepared as previously described (Pettijohn, 1967). RNA polymerase was purified by the method of Chamberlin and Berg (1962) with modifications as previously described (Pettijohn and Kamiya, 1967).

RESULTS AND DISCUSSION

RNA polymerase can be associated with template DNA in at least two distinct ways. It may be involved in a tertiary complex which includes the nascent RNA chain (Bremer and Konrad, 1964) or it may be passively bound in the absence of RNA synthesis (Kadoya et al, 1964; Pettijohn and Kamiya, 1967). Heparin inactivates RNA polymerase which is passively bound but has little effect on the tertiary complex (Walter et al, 1967). The effect of heparin on the RNA polymerase activity of a protein-DNA complex isolated from E. coli is shown in Fig. 1. In agreement with the results of Walter, et al (1967), RNA polymerase which was passively bound to T_4 DNA, T_7 DNA, or the protein-DNA complex was inactivated by the heparin, while enzyme which had initiated synthesis was insensitive to this polyanion. More than 80% of the RNA polymerase activity isolated with the protein-DNA complex was resistant to heparin

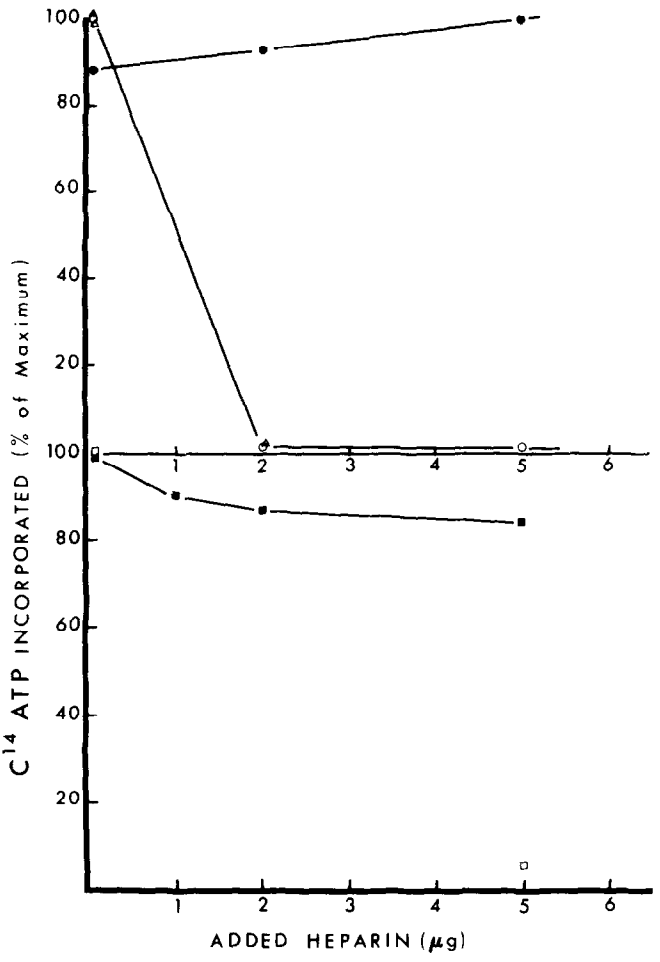

Fig. 1: Effects of heparin on in vitro RNA synthesis. (Upper) The reaction mixtures contained in 0.30 ml: 0.033 M tris (pH 7.8), 0.10 M KCl, 10 mM $MgCl_2$, 0.33 mM each of ATP, CTP, GTP and UTP, amounts of RNA polymerase and DNA as[2] given below, and variable amounts of heparin. Some of the mixtures (closed symbols) were incubated for 10 min at 37° to allow initiation of RNA synthesis before adding the heparin plus C^{14} ATP to 1 mC/m-mole; others (open symbols) were incubated first for 1 min at 37° in the absence of the triphosphates and heparin to permit binding of the polymerase (Pettijohn and Kamiya, 1967), then for 1 min in the presence of heparin, then triphosphates were added with C^{14} ATP at 1 mC/m-mole. Final incubations were for 10 min at 37°; the reactions were terminated by the addition of 5% trichloracetic acid and filtered through a Whatman GF/C glass filter which was dried and counted in a scintillation counter.

○ ○ ○ 2.8 μg T_4 DNA, 6 μg RNA polymerase, heparin added before initiation.

● ● ● 5.8 μg T_4 DNA, 3 μg RNA polymerase, heparin added after initiation.

△ △ 3.3 μg T_7 DNA, 3 μg RNA polymerase, heparin added before initiation.

▲ ▲ 3.3 μg T_7 DNA, 3 μg RNA polymerase, heparin added after initiation.

(Lower) Reaction mixtures as above, containing approximately 35 μg DNA in an E. coli (MRE 600) protein-DNA complex, pre-incubated with heparin for 1 min at 37° before the addition of triphosphates with C^{14} ATP at 1 mC/m-mole. Final incubation was 4 min at 37°.

■ ■ ■ No added RNA polymerase.

□ □ Complex incubated with 12 μg added RNA polymerase 1 min 37° before the addition of heparin or triphosphates. The C^{14} ATP incorporation which occurs in the absence of added polymerase is subtracted from this data.

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at concentrations which were sufficient to inactivate passively bound enzyme. This result suggests that the majority of the active RNA polymerase molecules present in the complex had initiated RNA synthesis prior to the isolation of the complex. Presumably, then, the RNA synthesis observed in vitro occurs predominantly as an extension of nascent chains which were initiated in vivo.

Radioactive RNA synthesized from the RNA polymerase-DNA complex has been purified and hybridized with E. coli DNA in competition with non-radioactive E. coli r-RNA (Fig. 2). The experiment was arranged so that the competitor r-RNA was prehybridized with the DNA and treated with RNase before the hybridization with the H^3-RNA was begun. This procedure eliminated the possibility that RNase sensitive complexes formed between DNA and the r-RNA may produce false competition (Gillespie, 1968). As shown in Fig. 2, the hybridization of approximately 20% of the hybridizable H^3-RNA could be prevented by prior hybridization of the DNA with r-RNA. By contrast, the same r-RNA preparation did not significantly compete with the hybridization of H^3-RNA synthesized in vitro from E. coli d-DNA templates. One should note in Fig. 2 that competition for the r-RNA complementary sites on the DNA was more than 90% complete at r-RNA inputs greater than 1.5 μg (defined by the availability for hybridization with H^3-r-RNA). This is near the r-RNA input where competition with the H^3-RNA synthesized from the complex was maximal. Thus, within the limits of accuracy of the data, H^3-r-RNA and 20% of the hybridizable H^3-RNA made from the complex, experience maximal competition from r-RNA at the same r-RNA/DNA ratio. It is concluded that a fraction of

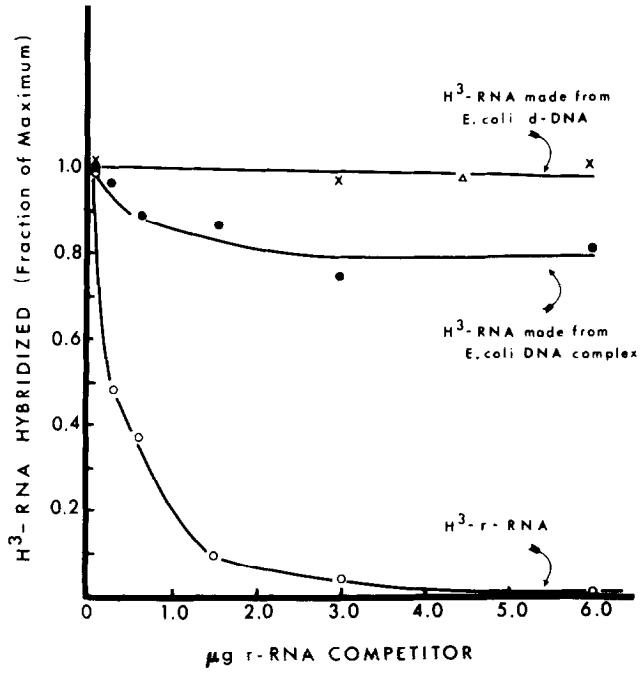

Fig. 2: Competition hybridization experiments. Nitrocellulose filters containing 110 μg E. coli d-DNA were incubated for 14 hours at 65° in 1.5 ml 6 X SSC containing 0.017% phenol plus variable amounts of non-radioactive r-RNA (2 parts 23S per 1 part 16S). They were then removed and soaked for 1.0 hr at 25° in 2 X SSC containing 10 μg/ml pancreatic RNase, batch washed in 3 changes of 6 X SSC and further washed by filtration with 6 X SSC. The filters were then incubated for 36 hrs at 57° in individual vials containing 6 X SSC with phenol as above plus

0 0 0 0.26 μg H^3-r-RNA composed of 2 parts 23S r-RNA per 1 part 16 S r-RNA. Maximum hybridization efficiency = 46%.

● ● ● 0.04 μg H^3-RNA synthesized from the RNA polymerase-DNA complex. Maximum hybridization efficiency = 30%.

X X X 0.06 μg H^3-RNA synthesized from E. coli d-DNA template. Maximum hybridization efficiency = 44%.

Δ Δ 0.014 μg H^3-RNA synthesized from E. coli d-DNA template. Maximum hybridization efficiency = 39%.

After treatment with RNase and washing as above, the filters were dried and counted. Blank filters which had been prepared in parallel with the DNA containing filters were present in each reaction vial during both hybridization and RNase steps. Each point plotted above has been corrected for the radioactivity retained on its corresponding blank.

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the RNA synthesized in vitro from the protein-DNA complex is indistinguishable in its hybridization properties from r-RNA.

In two repeats of the experiment shown in Fig. 2, a maximum of 18 ± 6% and 20 ± 7% of the hybridizable H^3-RNA made from the complex were prevented from hybridizing by prior incubation of the DNA with r-RNA. These experiments do not determine the actual fraction of the total RNA which has the hybridization properties of r-RNA. Since the hybridization efficiencies of r-RNA and other RNA species are probably not the same even in the absence of competitor and since the specific activities of radioactive labeling of the different RNA species may be different, there is no reason to expect a competition experiment such as that of Fig. 2 to directly reveal the true fraction of r-RNA present in an RNA preparation. However, these variables are open to experimental determination and are currently under investigation.

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