JOURNAL OF BACTERIOLOGY, Oct. 1979, p. 246-250
0021-9193/79/10-0246/05$02.00/0

Vol. 140, No. 1

Cyanobacterial Ribonucleic Acid Polymerases Recognize Lambda Promoters

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Received for publication 27 April 1979

We compared the initiation specificities in vitro of deoxyribonucleic acid-dependent ribonucleic acid polymerases purified from two cyanobacteria, *Fremyella diplosiphon* and *Anacystis nidulans*, and from *Escherichia coli*. A restriction fragment made from lambda deoxyribonucleic acid was used as a template. The cyanobacterial and *E. coli* ribonucleic acid polymerases recognized the same lambda promoters but exhibited different sensitivities to the inhibitor heparin, suggesting differences in the structure of the initiation complexes.

The ability of a DNA-dependent RNA polymerase to initiate transcription specifically at a site or sites (promoters) on a DNA template is defined as its initiation specificity or its selectivity (4). It is likely that RNA polymerase selectivity has been conserved during the evolution of procaryotes. For example, successful species conjugations, transformations, and transductions are common between gram-negative procaryotes, indicating the heterologous "foreign" DNAs are transcribed normally in new hosts. In a recent study, Wiggs et al. (10) have shown that RNA polymerases from a variety of gram-negative and gram-positive bacteria selectively transcribe from the same T7 promoter sites as the RNA polymerase from *Escherichia coli*. They concluded from these results that there has been strong selective pressure to maintain common promoter recognition sites in procaryotic systems. In this paper, we compare the ability of two RNA polymerases isolated from two species of cyanobacteria to initiate transcription specifically, in vitro, at two well-characterized bacteriophage promoters with the ability of the *E. coli* RNA polymerase to initiate at the same promoters.

The lambda chromosome contains two major early promoters: p_L, the promoter for the left operator (o_L), is responsible for the initiation of transcription of gene *N*; and p_R, the promoter for the right operator (o_R), which is responsible for the transcription of *tof* (*cro*) (Fig. 1). The lambda repressor binds within these operators and blocks transcription from both o_L and o_R.

Digestion of lambda DNA with the restriction endonuclease *Hae*III produces a 790-base pair (bp) fragment which carries p_R and p_M (the promoter for *c*I) (6). The fragment can be readily purified by preparative acrylamide gel electrophoresis and specifically binds *E. coli* RNA polymerase and bacteriophage lambda repressor protein. When used as an in vitro template for the *E. coli* RNA polymerase, the *Hae* 790 fragment directs the synthesis of at least two unique species of RNA which can be readily identified by electrophoresis on polyacrylamide. These two RNAs have been identified, in vitro, by Meyer et al. (6) as the *c*I and *tof* transcripts initiated at p_M and p_R, respectively. One RNA species (*tof*) starts at p_R and extends rightward 110 bp to the end of the fragment. The synthesis of this RNA is specifically inhibited by the lambda repressor protein. The other RNA (*c*I) is 300 bp in length and starts at p_M near p_R and extends to the left (Fig. 1). The synthesis of the *c*I RNA is also inhibited by the lambda repressor but at higher repressor concentrations.

We report here that RNA polymerases purified from the cyanobacteria *Fremyella diplosiphon* and *Anacystis nidulans* specifically initiate transcription, in vitro, at p_M (*c*I gene) and at p_R (*tof* gene) and, furthermore, that the transcription from these promoters is specifically inhibited by the bacteriophage lambda repressor protein.

MATERIALS AND METHODS

Enzymes and reagents. The restriction endonuclease *Hae*III (from *Haemophilus aegyptus*) was prepared as described by Maniatis et al. (5). Lambda repressor was a gift from Bob Sauer. The RNA polymerase from *E. coli* was prepared by the procedure of Berg et al. (3), and the cyanobacterial RNA polymerases were prepared by the method of Miller and Bogorad (7). [α-^{32}P]UTP (120 Ci/mol) was obtained from New England Nuclear Corp. Unlabeled nucleotides were purchased from Calbiochem.

DNA and restriction endonuclease fragments. Bacteriophage lambda *c*I857*sam*7 DNA was extracted

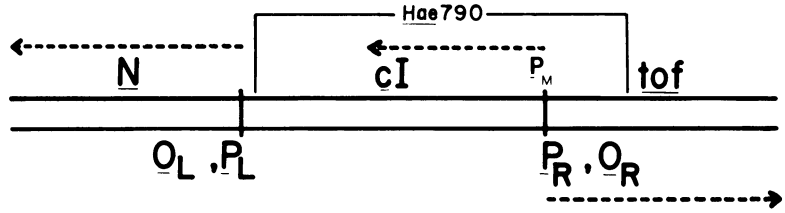

FIG. 1. *Diagram of the central portion of the lambda genome. Right and left promoters are indicated. The direction of transcription of genes is indicated by broken arrows.*

from CsCl-purified phage particles by lysis in 0.5% sodium dodecyl sulfate for 10 min at 65°C. After lysis, the solution was made 0.5 M in KCl, chilled for 15 min on ice, and spun at 10,000 × *g* for 15 min. The supernatant was then dialyzed against 10 mM Tris-hydrochloride (pH 7.5)–5 mM NaCl–1 mM EDTA for 48 h. Endonuclease *Hae*III restriction fragments of the purified DNA were prepared by polyacrylamide gel electrophoresis as described by Maniatis et al. (5).

Polyacrylamide gel electrophoresis. The 5% acrylamide (0.16% bisacrylamide)–7 M urea gels (16 by 20 cm) were poured and run, in the apparatus described by Ames (1), in a buffer comprised of 10 mM Tris-borate (pH 8.7) plus 2.5 mM EDTA (TBE buffer).

Transcription assays. Thirty-microliter transcription reaction mixtures of the *E. coli* RNA polymerase contained the following: 40 mM Tris-hydrochloride (pH 8.0), 10 mM $MgCl_2$, 50 mM KCl, 10% glycerol, 10 mM 2-mercaptoethanol, 15 nM DNA fragments, about 100 nM RNA polymerase, 200 μM ATP, 200 μM GTP, 10 μM CTP, 2.5 μM [α-^{32}P]UTP, and 250 μM heparin (Sigma Chemical Co.). In some assays, the concentration of UTP was increased to 6 μM by the addition of unlabeled nucleotide. The assay mixture for the cyanobacterial polymerases was the same, except that $MgCl_2$ was used at a concentration of 30 μM and KCl and heparin were omitted. For the repressor experiments, the DNA was preincubated with 0 to 25 molecules of repressor per molecule of DNA for 10 min at 37°C. In all other reactions, the preincubation was omitted and the DNA was incubated with the RNA polymerase for 10 min at 37°C. The reaction was then started by the addition of nucleoside triphosphates (with or without heparin), and the mixture was incubated for 20 min at 37°C. The reactions were halted by the addition of 0.5 ml of 2 M ammonium acetate–2 mM EDTA containing 80 μg of yeast tRNA (Schwartz Bioresearch) per ml as carrier. The samples were precipitated with 3 volumes of absolute ethanol and lyophilized. The lyophilized samples were suspended in 20 μl of TBE buffer and loaded directly onto polyacrylamide gels for electrophoresis.

Fingerprints. [^{32}P]RNAs, for analysis by fingerprinting, were first purified on 7 M urea polyacrylamide gels. After autoradiography, the appropriate bands were cut from the gel, sliced into small pieces, and soaked in 0.5 M ammonium acetate–10 mM magnesium acetate–0.1% sodium dodecyl sulfate for elution. The eluted RNA was precipitated with 3 volumes of ethyl alcohol. The RNA pellets were washed with cold alcohol and dried in a vacuum. This dried RNA was suspended in distilled water and lyophilized. The lyophilized RNA was suspended in 10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA–0.1 mg of T_1 RNase (Cabiochem) per ml. This reaction mixture was sealed within a capillary tube and incubated for 1 h at 37°C. After digestion, the reaction mixture was spotted onto DEAE paper, washed with distilled water, and eluted with triethylammonium bicarbonate (pH 9). The eluted RNA digest was then dried in a vacuum and suspended in a small volume (~5 μl) of distilled water.

Fingerprints of T_1-digested RNAs were made by electrophoresis in cellulose acetate and chromatography on polyethyleneimine, using the methods of Rose (9) and Barrell (2).

RESULTS

Comparison of *cI* and *tof* transcripts. We analyzed the transcripts produced in vitro by the various RNA polymerases by using the *Hae* 790 fragment of the lambda chromosome as a template. In the presence of 2.5 μM UTP, *E. coli* RNA polymerase produced three RNAs in vitro with this template. The largest RNA was a 300-bp transcript of the *cI* gene, the second was about 110 bp long, and the third was slightly shorter than 110 bp (Fig. 2A). When the UTP concentration in the transcription mixture was increased in 6 μM from 2.5 μM, the smallest transcript was not produced (Fig. 3A). A comparison of T_1 RNase fingerprints of the two smallest transcripts produced in the presence of 2.5 μM UTP shows that the smaller of these two RNAs was totally contained within the 110-bp species and was probably a "stutter" product (data not shown). Production of the stutter product of the 110-bp *tof* RNA has been reported previously (6).

RNA polymerase from *F. diplosiphon* produced the same complement of transcripts from the *Hae* 790 fragment as did the *E. coli* RNA polymerase in the presence of 2.5 μM (Fig. 2) and 6 μM (Fig. 3A and B) UTP. *A. nidulans* RNA polymerase also behaved like the *E. coli* enzyme in the presence of 6 μM UTP (Fig. 3A and C). (Its behavior in 2.5 μM UTP was not tested.)

The 110-bp *tof* transcripts produced by *F. diplosiphon* and *E. coli* RNA polymerases were examined in more detail. The 110-bp transcripts

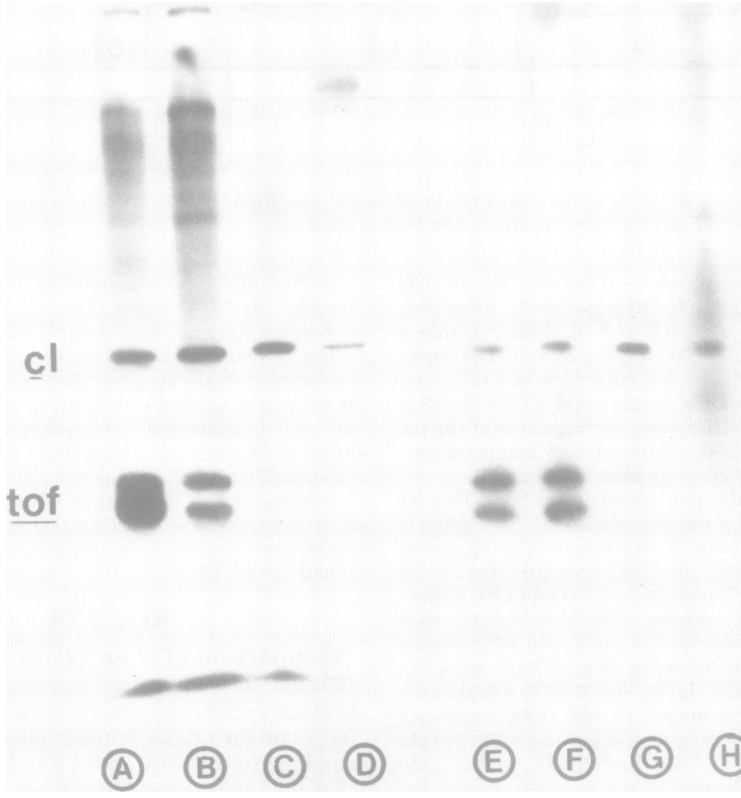

FIG. 2. *Titration of the specific transcription activity of the E. coli and F. diplosiphon RNA polymerases from Hae 790 with purified lambda repressor. Slots A through D were transcribed by the E. coli polymerase in the presence of 0, 2, 10, and 25 molecules of purified repressor, respectively, per template molecule; E through H were transcribed by the F. diplosiphon polymerase with the same concentrations of repressor. These transcriptions were done in the presence of 2.5 μM UTP.*

produced by the two enzymes were eluted from gel slices and digested with RNase T_1. The digestion products were separated by electrophoresis on cellulose acetate strips and chromatography in a second dimension on polyethyleneimine plates. The resultant fingerprints were indistinguishable from one another (Fig. 4).

Cyanobacterial enzymes also transcribed *c*I. However, the ratio of *tof* to *c*I transcription was lower than in the case of the *E. coli* RNA polymerase (Fig. 2 and 3).

The lambda repressor bound to the two operators on the lambda DNA, o_L and o_R, blocking the rightward transcription of *c*I and the leftward transcription of gene *N*. Transcription of *c*I is also inhibited by the lambda repressor, but somewhat higher repressor concentrations are necessary for this inhibition (6). Figure 2A through D shows the effect of adding increasing amounts of lambda repressor to the *E. coli* transcription mixture. Maximum repression of the *tof* transcript was found to occur at a ratio of about 10 repressor molecules to every molecule of template. This concentration of repressor had no effect on the amount of *c*I RNA transcribed. Higher concentrations of the lambda repressor (25 molecules per template molecule) were necessary to inhibit the transcription of *c*I. The transcription of *tof* RNA by the *F. diplosiphon* RNA polymerase (Fig. 2E through H) exhibited sensitivity to the lambda repressor similar to that of the *E. coli* RNA polymerase. The *c*I RNA made by the cyanobacterial polymerase, however, was not as strongly repressed as *E. coli* RNA polymerase by the high concentrations of the lambda repressor.

Effects of heparin. Heparin is a polyionic molecule which forms a complex with RNA polymerase and inactivates it. This polyanion inactivates free RNA polymerase faster than RNA polymerase which is bound to DNA. For this reason heparin has been used in previous studies

of RNA polymerase initiation specificity to reduce the nonspecific background by preventing reinitiation and initiation at promoter sites with weak affinity for the polymerase (8). We found that heparin, when added after the polymerase-DNA complex was formed, enhanced the specificity of transcription by *E. coli* polymerase but totally inhibited transcription by the *F. diplosiphon* and *A. nidulans* enzymes.

We used a heparin concentration of 250 μM in the assays using *E. coli* RNA polymerase. We tried using lesser concentrations of heparin (down to 25 μM) in the cyanobacterial reactions, but even low concentrations caused significant inhibition of the production of the RNA transcripts involved. Heparin was not necessary for faithful transcription of the lambda promoters by the cyanobacterial enzymes. In fact, transcription by the cyanobacterial polymerases was more specific (showed less background) without added heparin than that of the *E. coli* RNA polymerase with heparin present.

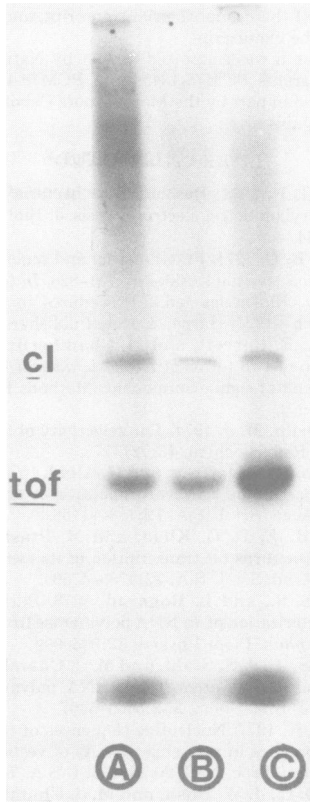

FIG. 3. *RNA transcripts made from the endonuclease HaeIII 790 fragment from the lambda chromosome by the RNA polymerases from (A) E. coli, (B) F. diplosiphon, and (C) A. nidulans. These transcriptions were done in the presence of 6 μM UTP.*

DISCUSSION

We showed that two cyanobacterial RNA polymerases specifically recognize the *tof* promoter (p_R) and probably the *c*I (p_M) promoter of phage lambda. Fingerprints of the RNase T_1 digestion products of cyanobacterial and *E. coli tof* transcripts were identical. Although we have not compared the fingerprints of the *E. coli c*I transcripts with an analogous cyanobacterial transcript, it is likely that they are also identical since the lambda repressor specifically inhibits the cyanobacterial *c*I transcripts.

One interesting difference between the cyanobacterial and bacterial initiation complexes was that a concentration of heparin used to prevent reinitiation in the *E. coli* transcription mixture totally prevented transcription by the cyanobacterial polymerase. Heparin can apparently displace the cyanobacterial RNA polymerase from the template because the RNA polymerase-DNA complex was allowed to form before the

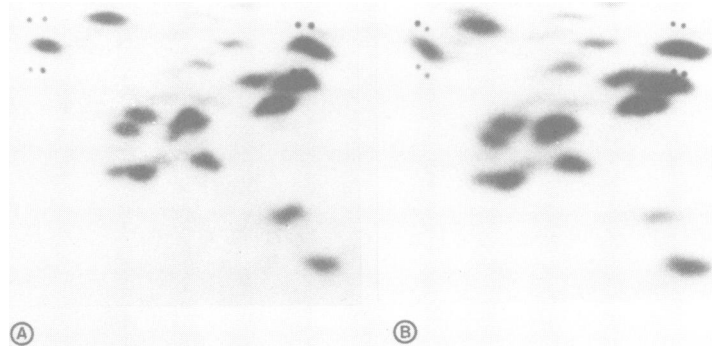

FIG. 4. *Fingerprints of the tof transcripts made by the polymerases from (A) E. coli and (B) F. diplosiphon.*

addition of heparin. This heparin inhibition gives no indication of the relative stability of the polymerase DNA complexes. Until recently, it was generally thought that stable polymerase-DNA complexes were resistant to heparin attack. Pfeffer et al. (8) have shown that all T7 promoter-polymerase complexes are attacked and that the rate of attack is not related to the stability of the complex or the strength of the promoters but, rather, is an indication of a difference in conformation of the polymerase enzyme in various complexes. They conclude from the fact that different polymerase promoter complexes are attacked by heparin at different rates that the polymerase conformation is altered by the structure of different promoter sites, these different conformations being attacked by heparin at different rates.

The differential heparin sensitivity of the tested cyanobacterial and bacterial RNA polymerases bound to a single promoter site may not be an indication of the relative stabilities of the polymerase-DNA complexes but probably indicates differences in the structure or conformation, or both, of the polymerases in this state.

We noted in our experiments that the relative promoter strengths of p_R and p_M are different when the different polymerases are used for transcription. In general, the *E. coli* polymerase sees p_M as a stronger promoter relative to p_R than do the cyanobacterial polymerases. Wiggs et al. (10) found that RNA polymerases from six bacterial genera all recognized the same major phage T7 promoters as did *E. coli* RNA polymerase. They were, however, able to detect dramatic differences in relative promoter strengths for each enzyme. They concluded that the relative strength of a promoter depends on the nature of the RNA polymerase as well as the nature of the template.

ACKNOWLEDGMENTS

We thank Barbara Meyer for her assistance in evaluating the transcription experiments, Jack Rose for preparing the fingerprints of the cyanobacterial transcript, and Rod Riedel for reading the manuscript.

This research was supported in part by National Science Foundation grants PCM75-13456 and PCM78-13231. It was also supported in part by the Maria Moors Cabot Foundation of Harvard University.

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