

The action of actinomycin D on the transcription of T7 coliphage DNA by *Escherichia coli* RNA polymerase

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An actinomycin D molecule bound to DNA sometimes stops the synthesis of RNA by *Escherichia coli* RNA polymerase. However, quite often, the bound antibiotic is released before the RNA polymerase detaches from the template DNA, so that the enzyme can resume, without interruption, the synthesis of the RNA chain.

It was previously shown that apurinic sites in the template strand of DNA were usually not a final stop to the progression of *Escherichia coli* RNA polymerase. If about 30% of the enzyme molecules did not go any further, 70% of them made only a pause before resuming the synthesis of an uninterrupted RNA transcript, placing a nucleotide in front of the apurinic site (Flamée & Verly, 1985).

Actinomycin bound to DNA is in equilibrium with a small pool of free antibiotic, so that, occasionally, a bound molecule detaches from DNA; according to Müller & Crothers (1968), at 37°C, the DNA/actinomycin D complex has a half-life of 260s. This half-life is shortened when RNA polymerase and the four deoxynucleoside triphosphates are added, as if the enzyme was able to scrape the antibiotic out of the double helix (Hyman & Davidson, 1970). The aim of this work was to investigate whether the techniques used successfully when the obstacle was an apurinic site might show that a bound actinomycin D molecule is not always a final stop to the progression of an RNA polymerase. I present data showing that, if an actinomycin D molecule bound to DNA sometimes definitively stops the synthesis of RNA by *E. coli* RNA polymerase, quite often the bound antibiotic is released before the RNA polymerase detaches from the template DNA, so that the enzyme can resume, without interruption, the synthesis of the RNA chain.

Materials and methods

T7 DNA

DNA from coliphage T7 was prepared as described by Verly *et al.* (1974); it contained an average of 0.3 break per strand (39936 nucleotides). The T7 DNA was stored at 4°C in 0.15M-NaCl/0.015M-EDTA, pH 7.0; just before utiliza-

tion, it was dialysed against 10mM-Tris/HCl, pH 7.9.

E. coli RNA polymerase

The preparation of the enzyme from *E. coli* Q13 was previously described (Flamée & Verly, 1985). The pure holoenzyme was stored at -20°C in 50mM-NaCl/10mM-Tris/HCl/10mM-MgCl₂/10mM-2-mercaptoethanol/0.1mM-EDTA/0.01% bovine serum albumin, pH 7.9, containing 50% (v/v) glycerol. Before use, it was diluted by steps with the same buffer containing 5% glycerol.

T7 RNA synthesis

(a) *Kinetic studies.* T7 DNA (0.96 pmol) and *E. coli* RNA polymerase holoenzyme (2.15 pmol) in 60 µl of 40mM-Tris/HCl/8mM-MgCl₂/10mM-mercaptoethanol/50mM-KCl/0.2% bovine serum albumin, pH 7.9 (preincubation buffer), were preincubated at 30°C for 10 min to allow the formation of the pre-initiation binary complex. The four nucleoside triphosphates [final concns. 0.4mM for ATP, GTP and CTP, and 0.1mM for UTP labelled with ³H in the uracil moiety (25 µCi/mmol)], heparin (final concn. 0.5 mg/ml), spermidine (final concn. 8mM) and KCl (final concn. 200mM) in water were added, giving a total volume of 100 µl.

The incubation was at 30°C and, at appropriate intervals, 5 µl samples were taken, placed on to GF/C Whatman glass-fibre filters, which were immersed in ice-cold 5% (w/v) trichloroacetic acid containing 10mM-sodium pyrophosphate. After 10 min, each filter was placed on a sintered-glass funnel and rinsed with 5 × 5 ml of 2% trichloroacetic acid/10mM-sodium pyrophosphate, and finally with 5 ml of ethanol. The filters were dried at 80°C for 20 min in an oven, then treated with 0.5 ml

of 1 M-HCl for 1 h also at 80°C to digest the nucleic acids. After addition of a scintillation solution, they were counted for radioactivity.

(b) *Preparation and analysis of T7 RNA.* T7 DNA (1.4 pmol) and RNA polymerase holoenzyme (3 pmol) were preincubated before addition of the four nucleoside triphosphates together with heparin. The procedure was the same as above, except that UTP was labelled with ^{32}P in the α position (125 mCi/mmol) and that the incubation was carried out at 37°C for 10, 20 or 30 min. The reaction was stopped by dipping the tubes in ice.

The unincorporated nucleoside triphosphates were removed by Sephadex G-50 gel filtration. The nucleic acids were precipitated with ethanol, then dissolved in 200 μl of 0.15 M-NaCl/0.015 M-EDTA, pH 7.0. Of this solution, 100 μl was layered on top of a 5–20% (w/v) sucrose gradient in 1 M-NaCl/10 mM-Tris/HCl/10 mM-EDTA, pH 7.0, in a 5 ml tube. After 120 min at 45000 rev./min in the SW50.1 rotor of a Beckman L5 centrifuge at 20°C, the tubes were emptied from the top and the radioactivity of the collected 100 μl fractions was determined.

Miscellaneous

The actinomycin D concentration was calculated from measurements of A_{445} by using $\epsilon = 22000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Ziffer *et al.*, 1968).

Experimental and results

E. coli RNA polymerase holoenzyme transcribes T7 DNA early genes. The three major promoters are placed very close to one another at the left end of the T7 genome map (Stahl & Chamberlin, 1977); a termination signal is located at 19% of the T7 genome map. Transcription from the major promoters to the terminator gives RNA molecules containing an average of 7000 nucleotides (Dunn & Studier, 1983).

Action of actinomycin D on the rate of T7 RNA synthesis

All incubations were carried out at 30°C. T7 DNA (0.96 pmol) was incubated with 0, 6, 12, 24 and 48 pmol of actinomycin D for 25 min in 55 μl of the preincubation buffer. Assuming random binding of the antibiotic to the T7 DNA, the average number of actinomycin D molecules sequestered in the part of the genome between the major promoters and the terminator were 0, 1, 2, 4 and 8 respectively. Addition of RNA polymerase holoenzyme (2.15 pmol) and, after a 10 min delay, of the nucleoside triphosphates and heparin is described in the Materials and methods section. Samples were taken after different times of

incubation and the radioactivity incorporated into RNA was measured.

Fig. 1 shows the results of the experiment. Without actinomycin D (situation *a*), the RNA radioactivity increased linearly with time until 11 min, which was the time needed for the enzyme to travel from promoter to terminator under the conditions of this particular experiment. After 11 min, the RNA radioactivity still increased linearly, but at a slower rate; the synthesis after 11 min was due to enzyme molecules that did not stop at the terminator. The ratio of the slope after 11 min to that before shows that 20% of the RNA polymerase molecules did not stop at the terminator.

The results were quite different when actinomycin D was present. To analyse them, I first made the assumption that an actinomycin D molecule bound to T7 DNA is a complete block to the progress of the RNA polymerase, by using the following equation (Flamée & Verly, 1985):

$$y = \frac{1}{px} (1 - e^{-px})$$

where y is the ratio of RNA synthesized in the

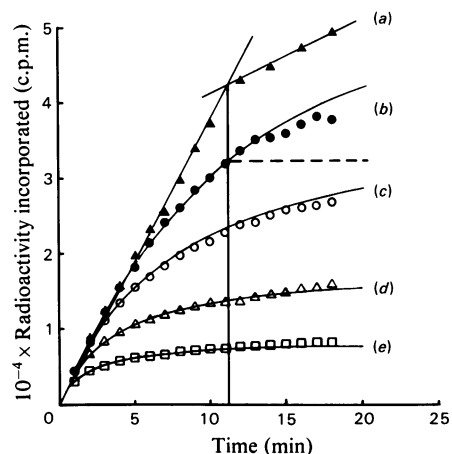


Fig. 1. Action of actinomycin D on T7 RNA synthesis. T7 DNA was preincubated with or without actinomycin D to have an average of 0 (\blacktriangle), 1 (\bullet), 2 (\circ), 4 (\triangle) or 8 (\square) molecules of the antibiotic between the major promoters and the terminator. RNA polymerase holoenzyme was then added to form a preinitiation complex before addition of ATP, GTP, CTP and ^{32}H UTP, together with heparin (0 min). Incubations were carried out at 30°C and samples taken at different times (min) to measure the radioactivity incorporated into RNA (c.p.m.). The theoretical curves were drawn for an average of 0.95 (b), 1.80 (c), 3.60 (d) and 8.00 (e) complete stops between the major promoters and the terminator, and a terminator of 0% efficiency (continuous lines) or 100% efficiency (broken line, situation b).

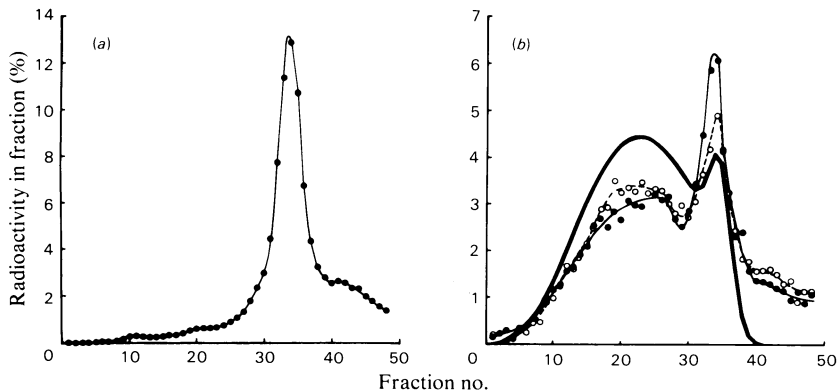


Fig. 2. Length of RNA synthesized in absence or presence of actinomycin D

The RNA was analysed by sedimentation on sucrose gradients, and the radioactivity of the collected fractions (% of total c.p.m.) plotted against the distance sedimented (fraction number). (a) T7 DNA and RNA polymerase holoenzyme were preincubated before addition of ATP, GTP, CTP, [α - 32 P]UTP and heparin, followed by a 10 min incubation at 37°C. The tail of the sedimentation profile corresponds to an average of 0.4 final stop between promoters and terminator. (b) T7 DNA containing an average of 3 bound actinomycin D molecules between major promoters and terminator was preincubated with RNA polymerase holoenzyme before addition of ATP, GTP, CTP, [α - 32 P]UTP and heparin. Samples were taken for analysis after 20 min (○) and 30 min (●) of incubation at 37°C. The bold continuous curve has been calculated for an average of 3.4 final stops (0.4 for background + 3.0 for the antibiotic) between promoters and terminator.

presence and absence of actinomycin D when the unimpeded enzyme had added x nucleotides to the growing RNA chain, and p is the probability that the RNA polymerase will be stopped at any nucleotide of the template DNA. The average number of complete blocks between promoter and terminator, $n = 7000 p$.

The experimental data up to 11 min were used to calculate n by successive approximations with a computer. Fig. 1 shows that the best theoretical curves fit the experimental data rather well, and the calculated n values (see legend of Fig. 1) are in good agreement with the average numbers of actinomycin D molecules in the segment considered for transcription.

The situation after 11 min is different. The theoretical curves were drawn supposing that the terminator was completely inefficient, except for the broken horizontal line in part (b), which represents a theoretical situation with a 100% efficient terminator. If a bound actinomycin D molecule were always a final block to the enzyme progression, the experimental results should lie between these two extremes. This is surely not the case with situations (d) and (e), where the experimental points are above the theoretical curves assuming the absence of terminator.

Action of actinomycin D on the length of the synthesized RNA

The experiment was carried out with 0 or 3 bound actinomycin D molecules between major

promoters and terminator. The reaction was stopped after 10 min (without actinomycin D), 20 or 30 min (with actinomycin D) at 37°C, and the RNA synthesized was analysed on 5–20% sucrose gradients as described in the Materials and methods section.

Fig. 2 gives the results without (a) and with (b) actinomycin D. The peak of complete RNA molecules (7000 nucleotides) is centred on fraction 34. Even with the antibiotic, although the incubation times (20 and 30 min) were much greater than the 5 min needed at 37°C for the unimpeded enzyme to go from promoter to terminator (results not shown), there were not many RNA molecules longer than 7000 nucleotides. On the other hand, the fraction of complete RNA molecules (7000 nucleotides) increased, whereas the fraction of incomplete molecules decreased between 20 and 30 min.

Discussion

I have studied the action of actinomycin D on the rate of transcription of the T7-phage DNA early region by *E. coli* RNA polymerase holoenzyme and also on the length of the RNA synthesized.

In all experiments, the ratio RNA polymerase/T7 DNA was below 3, so that only the three major promoters were used. A preincubation of T7 DNA and RNA polymerase enabled me to synchronize the initiation when the nucleoside

triphosphates were subsequently added; heparin was used to prevent re-initiation.

The kinetic experiments were done at 30°C to slow down the enzyme progression and determine more experimental points. In absence of actinomycin D, the RNA polymerase took 11 min to go from promoter to terminator; about 20% of the enzyme molecules were not stopped, and continued to transcribe the DNA past the terminator (see also Chamberlin *et al.*, 1979). In the presence of actinomycin D, the experimental points giving the amount of RNA synthesized as a function of time were in good agreement, during the first 11 min, with theoretical curves drawn on the assumption that bound actinomycin D molecules irreversibly block the progression of the enzyme. These results could be considered as a check of the value of the method used to analyse the inhibition of RNA synthesis.

But, after 11 min, when the number of bound actinomycin D molecules was high, the amount of RNA synthesized was greater than what the model predicted, even with the additional assumption that the terminator was completely inefficient. This result could be correlated with the possibility for the DNA/actinomycin D complex to dissociate so that, after a pause, the RNA polymerase could proceed further: after 11 min, the RNA synthesized by retarded enzyme molecules would add to the RNA synthesized by the enzyme molecules that were not stopped by the terminator.

To check this interpretation, I have analysed the length of the RNA molecules synthesized at 37°C after a delay longer than the 5 min needed, at this temperature, for an unimpeded enzyme to travel from promoter to terminator. In the presence or absence of actinomycin D, there were few molecules longer than 7000 nucleotides, suggesting that the large delayed RNA synthesis observed in the kinetic experiments in the presence of actinomycin D was not due to a decreased efficiency of the terminator. On the other hand, it was remarkable to observe that, in the presence of actinomycin D, RNA molecules of 7000 nucleotides were still completed between 20 and 30 min. Moreover, after 30 min, the fraction of completed RNA molecules (7000 nucleotides) was twice that predicted on the assumption that a bound actinomycin D molecule is a final block to the RNA polymerase, as shown by the theoretical curve drawn in Fig. 2(b). A bound actinomycin D molecule is thus not an

irreversible block; when it clears the way, the RNA polymerase can resume, without interruption, the synthesis of the RNA chain.

This does not necessarily mean that all the RNA chains will eventually be completed. If one compares the situation after 30 min at 37°C, when there is an average of 3 bound actinomycin D molecules in the promoter-terminator segment, with that in the absence of the antibiotic, one sees many more incomplete RNA chains when actinomycin D is present. The profile in the absence of the antibiotic, after a time longer than that needed for the enzyme to go from promoter to terminator, already shows a tail of unfinished RNA molecules (Fig. 2a) corresponding to an average of 0.4 block between promoter and terminator. With the antibiotic (Fig. 2b), the problem is more difficult to analyse, because we do not know whether more RNA chains would not have been completed after 30 min; but the profile at 30 min has a tail of unfinished RNA molecules much larger than that observed in absence of the antibiotic, and it is unlikely that, given more time, the two profiles would have been identical. In other words, the RNA polymerase sometimes either paused before a permanently bound actinomycin D molecule, or detached from the DNA before the antibiotic had a chance to clear the way.

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References

- Chamberlin, M. J., Nierman, W. C., Wiggs, J. & Neff, N. (1979) *J. Biol. Chem.* **254**, 10061-10069
- Dunn, J. J. & Studier, F. W. (1983) *J. Mol. Biol.* **166**, 477-535
- Flamée, P.-A. & Verly, W. G. (1985) *Biochem. J.* **229**, 173-181
- Hyman, R. W. & Davidson, N. (1970) *J. Mol. Biol.* **50**, 421-438
- Müller, W. & Crothers, D. M. (1968) *J. Mol. Biol.* **35**, 251-290
- Stahl, S. J. & Chamberlin, M. J. (1977) *J. Mol. Biol.* **112**, 577-601
- Verly, W. G., Crine, P., Bannon, P. & Forget, A. (1974) *Biochim. Biophys. Acta* **349**, 204-213
- Ziffer, H., Yamaoka, K. & Mauger, A. B. (1968) *Biochemistry* **7**, 996-1001