DIFFERENTIAL INHIBITION OF TRANSCRIPTIONS CATALYZED BY T7-SPECIFIC AND *ESCHERICHIA COLI* RNA POLYMERASES BY BLEOMYCIN

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The effect of bleomycin on *in vitro* T7 DNA transcriptions catalyzed by phage T7-specific and *Escherichia coli* RNA polymerases was investigated. It was observed that T7 RNA polymerase was more readily inhibited by the antibiotic than *E. coli* RNA polymerase. The possible mechanism which would explain the differential inhibition of the transcription was discussed.

Bleomycin, a water-soluble basic glycopeptide antibiotic, produced by *Streptomyces verticillus*[1,2], exhibits antitumor and antibacterial activity[3]. It has been reported by SUZUKI *et al.*[4] that bleomycin inhibits DNA and protein syntheses in intact *Escherichia coli*, EHRLICH carcinoma and HeLa cells, but RNA synthesis is not suppressed significantly by the antibiotic. Bleomycin interacts directly with DNA molecule and remains bound to DNA. Two types of binding of bleomycin to DNA are known: one is binding to DNA modified with sulfhydryl group leading to strand scission and the other is binding to DNA with no sequential reaction in the absence of the sulfhydryl group[5].

Recently, a new type of RNA polymerase was isolated from phage T7-infected cells of *E. coli*[6]. This enzyme is distinct from the host *E. coli* RNA polymerase in its template specificity. The former synthesizes RNA only when T7 DNA or synthetic poly dG:dC was used as the template[6,7]. Analysis of the purified T7-specific RNA polymerase using SDS acrylamide gels reveals a single protein component of molecular weight of 107,000. In particular, it is found that protein components corresponding to the β, β', σ, and α bands of bacterial RNA polymerase are absent. In this study the effect of bleomycin on the *in vitro* T7 DNA transcriptions catalyzed by T7-specific and *E. coli* RNA polymerases was studied, and T7 RNA polymerase was found to be more sensitive to bleomycin inhibition than *E. coli* RNA polymerase.

Materials and Methods

Bleomycin: Lot 7-52G81G82As; DM-A_2(0.6%), A_2(67.9%), B_2(30.3%), A_5(1.2%), copper (<0.01%)) prepared by Nihon Kayaku Co., Tokyo, was supplied generously from Dr. Y. OKAMI of the Institute of Microbial Chemistry, Tokyo. The antibiotic was dissolved in water and stored at −20°C.

Deoxyribonucleic acid. T7 DNA was prepared according to THOMAS and ABELSON[8]. Phage T7 was a gift from J. TOMIZAWA of the National Institutes of Health, U. S. A. Cold and ^{3}H-labeled nucleo side triphosphates were purchased from Calbiochem Co., Los Angeles, and Schwartz Bio-Research, New York, respectively.

Enzymes. T7-Specific RNA polymerase was prepared from phage T7-infected cells of *E. coli* B

as described by CHAMBERLIN *et al.*[8] and the preparation of phosphocellulose fraction (Fraction F in their method) was used. This preparation contained no ribo- or deoxyribonuclease activity and had a specific activity of 15,000 units/mg protein. *E. coli* RNA polymerase holoenzyme was prepared from *E. coli* B cells by the modified method of CHAMBERLIN and BERG[9] as described previously[10]. This preparation showed a sedimentation coefficient of 22S and had a specific activity of 3,000 units/mg protein. One unit of polymerase activity is the amount of enzyme which incorporates 1 mμ mole of GMP into acid-insoluble fraction in 1 hour at 37°C in the assays indicated below.

Enzyme assays. T7-Specific RNA polymerase: 0.25 ml of reaction mixture contained 10 μ moles Tris-HCl (pH 8.0), 5 μ moles $MgCl_2$, 5 μ moles KCl, 3 μ moles β-mercaptoethanol, 100 μg bovin serum albumin, 5 μg T7 DNA, 100 mμ moles each of four ribonucleoside triphosphates (^{3}H-labeled GTP 3.0×10^6 cpm/μ mole, was used as the radioactive marker), indicated amounts of T7 RNA polymerase and indicated amounts of bleomycin. *E. coli* RNA polymerase: 0.25 ml of reaction mixture contained 10 μ moles Tris-HCl (pH 8.0), 1 μ mole $MgCl_2$, 5 μ moles KCl, 3 μ moles β-mercaptoethanol, 250 mμ moles $MnCl_2$, 5 μg T7 DNA, 100 mμ moles each of four ribonucleoside triphosphates (^{3}H-labeled GTP 3.0×10^6 cpm/μ mole was used as the radio active marker), indicated amounts of *E. coli* RNA polymerase and indicated amounts of bleomycin. Reaction mixtures were incubated at 37°C for indicated period and the reaction was terminated by addition of 1.0 ml of 10% ice-cold trichloroacetic acid. Radioactivity of the acid-insoluble fraction was measured in a Beckman DPM 100 type liquid scintillation spectrometer.

Sucrose density gradient centrifugation. DNA samples were layered on 4.5 ml of a gradient (5~20% sucrose supplemented with 10 mM Tris-HCl (pH 7.5) and 0.1 M NaCl) and subjected to centrifugation in a Hitachi model 55PA ultracentrifuge using RPS 40 rotor at 39,000 rev/min for 3 hours at 1°C. Then, 0.18 ml fractions were collected from the bottom of the tube and A_{260} nm was measured in a Hitachi 124 spectrophotometer.

Results and Discussion

Bleomycin inhibits both T7 DNA transcriptions catalyzed by T7-specific and *E. coli* RNA polymerases with the former enzyme being more sensitive (Fig. 1). This difference in response was not dependent on concentration of enzyme present since a 100-fold variation in enzyme concentraton (0.1~10 units) produced no significant differences in amounts of RNA synthesized.

Addition of bleomycin to the T7 polymerase reaction at different times did not alter CMP incorporation into RNA (Table 1). This indicates little or no effect of the antibiotic on the initiation of synthesis of RNA. This is also supported by the results in Fig. 3.

Incubation of T7 DNA with increasing concentrations of bleomycin in 50 mM Tris-HCl (pH 7.5)-2 mM β-mercaptoethanol at 37°C for 60 minutes and analysis of neutral sucrose gradient centrifugation suggested increasing fragmentation of the DNA (Fig. 2). If the centrifugation was carried out in alkaline (not shown in text), the fragmentation of DNA was markedly enhanced. This supports the conclusions of SUZUKI *et al.*[5] that bleomycin produces the single-strand scission of DNA in the presence of β-mercaptoethanol and the scission at the adjacent sites on interstrand introduces the fragmentation of DNA.

Fig. 3 illustrates that the difference in amounts of inhibition and rates of RNA synthesis was observed when bleomycin was supplemented in two polymerases reactions. T7-Polymerase (a) was

Fig. 1. Effect of bleomycin on T7 DNA transcriptions catalyzed by T7-specific and *E. coli* RNA polymerases as a function of amount of antibiotic

The RNA syhtheses catalyzed by the two RNA polymerases were investigated as described in Materials and Methods. Five units each the enzymes and indicated amounts of the antibiotic were used. The reactions were carried out for 15 minutes.

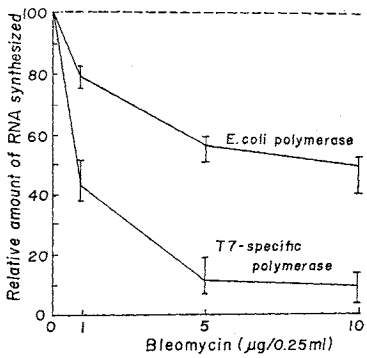

Fig. 2. Sedimentation patterns of the bleomycin treated DNA's

T7 DNA (100 μg/ml) was incubated at 37°C for 60 min with various concentrations of bleomycin in 50 mM Tris-HCl (pH 7.5)-2 mM β-mercaptoethanol. The incubation mixtures (0.1 ml) were subjected to centrifugation as described in Materials and Methods. 0.18 ml fractions were collected and $A_{260\,nm}$ was measured.

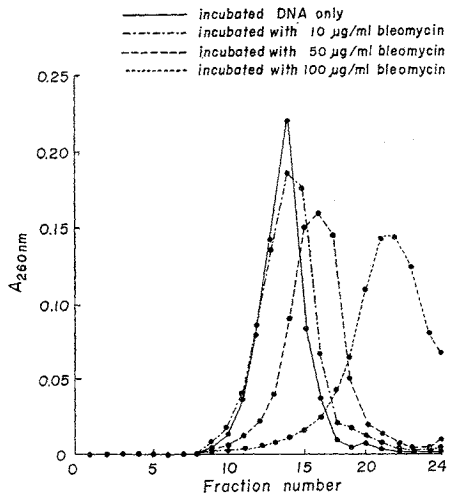

Table 1. Inhibition of T7 RNA polymerase action by bleomycin as a function of time when beolmycin is added to the reaction

The reaction mixtures were prepared as described in Materials and Methods except that the fixed amount of antibiotic (10 μg) and polymerase (5 units each) were added at the different times. After preincubation for 30 minutes, reaction mixture was adjusted to contain ATP, GTP, UTP and ^{3}H-labeled CTP (3.0×10^6 cpm/μ mole) and incubated for an additional 15 minutes. The figures in the parentheses are the percentages of the CMP incorporation relative to a control reaction

Reactants added at 0 min	15 min	30 min	Incorporation of (^{3}H) CMP into RNA products, cts/min
DNA + ATP, GTP + Polymerase	——→	→ (^{3}H) CTP, UTP →	3,266 (100)
DNA + ATP, GTP + Bleomycin, Polymerase	——→		349 (10.7)
DNA + ATP, GTP + Bleomycin	→Polymerase →		300 (9.2)
DNA + ATP, GTP + Polymerase	→Bleomycin →		395 (12.1)
ATP, GTP + Bleomycin, Polymerase	→DNA→		304 (9.3)

more sensitive, and the rate of synthesis was markedly reduced in the early period. Comparatively low extent of inhibition was observed with *E. coli* polymerase (b), and the rate of synthesis was constant for at least 2~5 minutes.

Table 2 presents the analysis of the early phases of RNA synthesis catalyzed by E. *coli* polymerase with a bleomycin-treated DNA template.

Fig. 3. Kinetics of the RNA syntheses catalyzed by T7-specific and *E. coli* RNA polymerases on the bleomycin treated DNA's

Reaction mixtures were prepared as described in Materials and Methods. Seven units each of the polymerases and 5 μg each of DNA treated with various concentrations of bleomycin were used. The mixtures were incubated for the indicated periods.

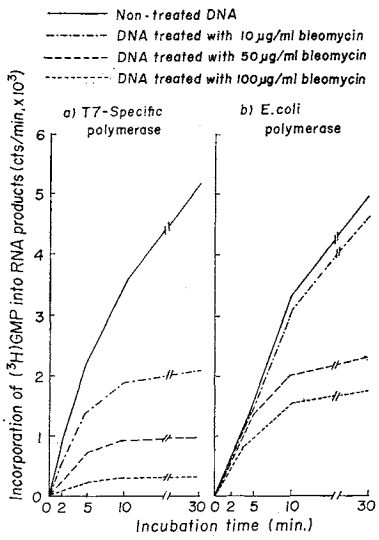

Table 2. Early phase of RNA syntheses catalyzed by *E. coli* RNA polymerase on the bleomycin-treated DNA's

The reaction mixtures were prepared according to Fig. 3 except that highly radioactive (^{3}H) GTP (1.0×10^7 cpm/μmole) was used. Incubation was carried out for the indicated periods (15, 30, 60 seconds)

DNA	Incorporation of (^{3}H) GMP into RNA products, cts/min.		
	15 sec.	30 sec.	60 sec.
Control	251	494	1,027
Treated with 10 μg/ml	—	—	1,056
50 μg/ml	243	501	983
100 μg/ml	260	480	1,085

Recently, VOGT[11] and HINKLE *et al.*[12] have reported that *E. coli* RNA polymerase core enzyme (lacking sigma factor) has a requirement for single strand breaks for RNA synthesis. However, since the RNA polymerase used in this paper was a holoenzyme, there was not observed as shown in Table 2 the stimulation of synthesis on account of the production of new initiation sites produced by the antibiotic treatment. Not shown in the table, the 2~3-fold stimulation was observed using the core enzyme in place of the holoenzyme. Thus, it seems that the results shown in Fig. 3(b) do not exhibit the co-existence of both stimulation and inhibition of the RNA synthesis.

In conclusion, observations described in this report are interpreted as follows: Inhibition in the case of *E. coli* RNA polymerase should seem to be mainly due to the decrease in size of template DNA. In contrast the inhibition in the case of T7 RNA polymerase would appear to be due to the interruption of the chain elongation at a site of bleomycin binding. SUZUKI *et al.*[5] reported that molar binding ratio of DNA nucleotide to bleomycin A_2 is less than 350 : 1 when 250 μg/ml of *E. coli* DNA and 100 μg/ml of the antibiotic are incubated at 37°C for 2 hours. If the five to ten phosphodiester bonds per second are formed in both RNA-synthesizing systems under conditions in Fig. 3, the enzyme should reach the site of bleomycin binding within one minute. Interestingly, FALASCHI and KORNBERG[13] reported previously that there was a distinction between the inhibition of the DNA and RNA polymerases obtained from *E. coli* by phleomycin. Phleomycin is related to bleomycin chemically, and one of several possible mechanisms which would explain these results assumes that DNA polymerase was interrupted when it reached a site of phleomycin binding, RNA polymerase by contrast could skip a region of phleomycin attachment.

Biologically, it is very interesting that bleomycin exhibits the distinctive effect between the host and phage-induced RNA synthesizing enzymes. In the preliminary data, bleomycin more effectively inhibits the production of phage T7 than T4. It is known that the infection with T4 does not induce the new phage specific RNA polymerase like T7.

Actinomycin D, an extensively studied inhibitor of RNA synthesis strongly inhibits the RNA syntheses catalyzed by both polymerases (unpublished observations).

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