INHIBITION OF INITIATION OF DNA-DEPENDENT RNA SYNTHESIS BY AN ANTIBIOTIC B44P

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An antibiotic B44p isolated in our laboratory from culture filtrates of a streptomyces is a mixture of pigment antibiotics which were identified as streptovaricins (Yamazaki and Umezawa, 1967). This substance exerts a preferential effect on RNA synthesis in S. aureus and in E. coli (Yamazaki et al., 1967) and inhibits the DNA-dependent RNA polymerase (E C 2.7.7.6) reaction of E. coli (Mizuno et al., 1967). It does not bind to the DNA template (Mizuno et al., 1967). On the other hand, the antibiotic B44p does not inhibit RNA synthesis by the soluble DNA-dependent RNA polymerase which is extracted from Ehrlich ascites cells (Mizuno et al., 1967).

In this communication, we report further on the mechanism of the inhibition of the RNA polymerase reaction of E. coli by B44p.

RNA synthesis by DNA-dependent RNA polymerase can be separated into three distinct processes: (1) binding of enzyme to specific sites on the DNA template to form a DNA-enzyme complex (Kadoya et al., 1964; Fox et al., 1965; Richardson, 1966; Jones and Berg, 1966; Ishihama and Kameyama, 1967), (2) initiation of RNA synthesis by reaction of the DNA-enzyme complex with purine nucleotides to form a stabilized initiation complex (Maitra and Hurwitz, 1965; Anthony et al., 1966), and (3) polymerization of four kinds of nucleotides into RNA chain under the direction of the DNA template (Bremer and Konrad, 1964; Ishihama and Kameyama, 1967).

The effect of B44p was studied on the each of the above steps of RNA synthesis in order to clarify which step is blocked by the antibiotic. We obtained evidence that B44p inhibits neither the formation of the DNA-enzyme complex nor the polymerizing process but rather, it inhibits the initiation of RNA synthesis. Therefore, B44p has a unique mode of action in its inhibition of RNA synthesis by DNA-dependent RNA polymerase.

MATERIALS AND METHODS : DNA-dependent RNA polymerase was prepared from E. coli B by the method of Chamberlin and Berg (1962) except for the disruption of the cells through a French press. The specific activity of the partially purified enzyme soon after the preparation was approximately 500 mμmoles of CMP incorporated/mg protein/10 minutes. Calf thymus DNA was obtained from Sigma Chemical Co. ^{32}P-labeled DNA was prepared by the method of Saito and Miura (1963) from E. coli B grown in Tris-casamino acid-glucose medium (Kozinski and Szybalski, 1959) containing ^{32}P-orthophosphate. ^{3}H-CTP and $\beta\gamma$-^{32}P-GTP were obtained from Schwarz Bioresearch, Inc.

The assay of RNA polymerase activity was described in a previous paper (Mizuno et al., 1967). The assay of enzyme binding to DNA was carried out by a membrane filter technique of Jones and Berg (1966). The millipore membrane filters used were of type HA(pore size 0.45μ), plain, white, 13 mm diameter.

The radioactivity of ^{32}P-DNA retained on filters was counted by an endwindow GM-counter after the filters were dried on planchets. The radioactivity of ^{3}H-CMP and $\beta\gamma$-^{32}P-GTP incorporated into acid-insoluble fractions was counted in a windowless gas-flow counter.

RESULTS AND DISCUSSION : Effect of B44p on the formation of DNA-enzyme complex. Native DNA or RNA polymerase is readily filterable through Millipore membrane filters, whereas the DNA-enzyme complex is quantitatively retained by the filter (Jones and Berg, 1966). By this membrane filter method the effect of B44p was examined on the binding of enzyme to DNA to form DNA-enzyme complex

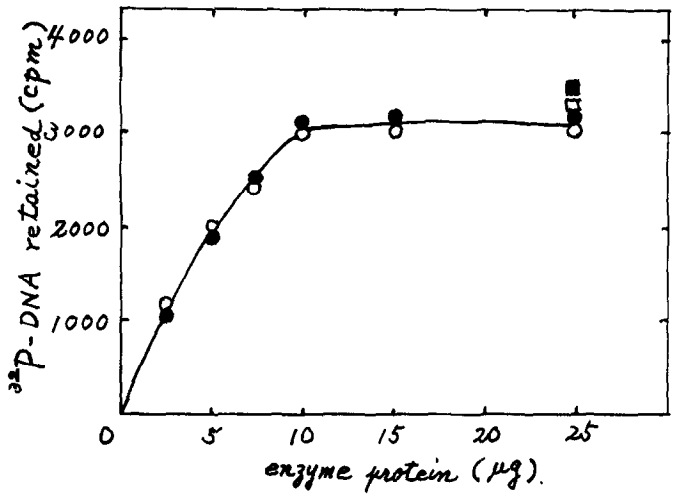

Fig. 1. Effect of B44p on RNA polymerase-dependent retention of ^{32}P-labeled E. coli DNA on membrane filters. The reaction mixture (0.2 ml) contained 1.2 μg of ^{32}P-labeled DNA (approximately 4,600 cpm), Tris-HCl, pH 8.0, 10 μmoles; $MgCl_2$, 1 μmole; β-mercaptoethanol, 2 μmoles; and indicated amounts of enzyme protein. Incubation was at 37°C for 1 min. (o) Control, (●) B44p 2 g/ml, (□) Control (2 min. incubation), (■) B44p (2 μg/ml) was added after ^{32}P-DNA and enzyme were incubated for 1 min. and the mixture was further incubated for 1 min. (test for the dissociation of the complex by B44p).

(Fig. 1). When 1.2 μg of ^{32}P-labeled E. coli DNA was used, the amount of the retained fraction on the filter was proportional to the amount of the enzyme added up to 10 μg protein. In the case of heat-denatured enzyme (75°C, 5 min.) there was almost no retention of ^{32}P-labeled DNA (not indicated in Fig. 1). In the presence of B44p (2 μg/ml), the amount of ^{32}P-labeled DNA retained was not affected at all. This indicates that B44p does not inhibit the binding of the enzyme to DNA to the form DNA-enzyme complex. Furthermore B44p could not dissociate the pre-formed complex (Fig. 1).

Effect of B44p on the initiation and polymerization steps of RNA synthesis. As reported in a previous paper (Mizuno et al., 1967), when the antibiotic B44p is added to the complete reaction mixture for RNA synthesis by E. coli

RNA polymerase at 0°C and thereafter the reaction is carried out at 37°C, strong inhibition of overall RNA synthesis is observed. Little inhibition, however, is seen if the antibiotic is added after the incubation of the complete reaction mixture at 37°C for 2 minutes. These experimental results suggest that the antibiotic B44p does not inhibit the polymerization process but inhibits initiation of RNA synthesis.

Initiation and subsequent polymerization of nucleotides into RNA chains by DNA-dependent RNA polymerase proceeds through a mechanism in which the first purine nucleotide incorporated into RNA chain retains its triphosphate moiety (Maitra et al., 1965; Maitra and Hurwitz, 1965; Bremer et al., 1965). Therefore, in order to provide direct evidence that B44p inhibits initiation of RNA synthesis, the effect of this antibiotic was examined on the incorporation of $\beta\gamma$-^{32}P-labeled GTP at 5'-termini of RNA chains and was compared with the effect on the incorporation of ^{3}H-CMP which represented total RNA synthesis (Table I). The results showed clearly that in both cases with native and heat-denatured calf thymus DNA as template, 5 μg/ml of B44p markedly inhibited the incorporations of $\beta\gamma$-^{32}P-GTP and ^{3}H-CMP. The rate of the inhibition by B44p of $\beta\gamma$-^{32}P-GTP incorporation could fully explain the inhibition of overall RNA synthesis represented by ^{3}H-CMP incorporation.

We further obtained the data which indicated that pre-incubation of the enzyme with DNA in the presence of a purine nucleotide protected the subsequent ^{3}H-CMP incorporation from B44p action (Table II). Namely, when the enzyme was pre-incubated (37°C, 2 min.) only with DNA, B44p (2 μg/ml) showed 62.5 % inhibition of ^{3}H-CMP incorporation in the subsequent 10 minute incubation (expt. 2). However, when the enzyme was pre-incubated with DNA in the presence of ATP or ATP plus UTP, the inhibition by the antibiotic was decreased to 21.5 % and 19.0 %, respectively (expt. 3 and 6). The pre-incubation with GTP also decreased the inhibition (expt. 4) but the pre-incubation with UTP showed only slight decrease of the inhibition (expt. 5). These results indicate that the polymerization process is

Table I. Inhibitory Effect of B44p on Incorporations of $\beta\gamma$-^{32}P-GTP and ^{3}H-CMP

	$\beta\gamma$-^{32}P-GTP ($\mu\mu$moles)	^{3}H-CMP ($m\mu$moles)
With native DNA		
Control	1.49	3.78
+ B44p, 5 μg/ml	0.20 (86.4*)	0.67 (82.3)
With heat-denatured DNA		
Control	4.38	1.42
+ B44p, 5 μg/ml	0.62 (85.9)	0.26 (81.7)

* The numbers in parentheses represent percent inhibition. The reaction mixture (0.3 ml) contained Tris-HCl, pH 8.0, 15 μmoles; β-mercaptoethanol, 3.6 μmoles; $MgCl_2$, 1.2 μmoles; $MnCl_2$, 0.3 μmole; native or heat-denatured calf thymus DNA, 37 μg; ATP, GTP, UTP and CTP, 50 $m\mu$moles each, $\beta\gamma$-^{32}P-GTP (5×10^5 cpm/$m\mu$mole) substituted for GTP for measurement of triphosphate termini or ^{3}H-CTP (3000 cpm/$m\mu$mole) substituted for CTP for measurement of the total RNA synthesis, 12 μg of RNA polymerase and where indicated B44p, 5 μg/ml. Incubation was at 37°C for 10 min. The assay of $\beta\gamma$-^{32}P-GTP incorporated was carried out by the procedure of Maitra and Hurwitz (1965).

insensitive to B44p action after the stabilized initiation complex is formed.

Actinomycin D does not affect the binding of RNA polymerase to DNA template and has less effect on the initiation than on the polymerization process (Richardson, 1966). Proflavine and high concentration of KCl or $(NH_4)_2SO_4$ affect the binding of the enzyme to DNA resulting in inhibition of the initiation and the subsequent polymerization process (Richardson, 1966; Anthony et al., 1966; Maitra et al., 1966).

As demonstrated by the data described here, the antibiotic B44p (streptovaricins) has no effect on the binding of RNA polymerase to the

Table II. Effect of B44p on ^{3}H-CMP Incorporation under the Various Conditions of Pre-incubation of RNA polymerase

Expt. no.	Pre-incubation (37°C, 2 min.)	Incubation (37°C, 10 min.)	^{3}H-CMP (mμmoles)	Inhib. (%)
1.		Enz,DNA,AUGC*	1.26	
		Enz,B44p,DNA,AUGC*	0.47	62.7
2.	Enz, DNA,	AUGC*	0.96	
	Enz, DNA,	B44p, AUGC*	0.36	62.5
3.	Enz, DNA, A,	GUC*	1.21	
	Enz, DNA, A,	B44p, GUC*	0.95	21.5
4.	Enz, DNA, G,	AUC*	1.20	
	Enz, DNA, G,	B44p, AUC*	0.80	33.3
5.	Enz, DNA, U,	AGC*	1.02	
	Enz, DNA, U,	B44p, AGC*	0.46	54.9
6.	Enz, DNA, AU,	GC*	1.21	
	Enz, DNA, AU,	B44p, GC*	0.98	19.0

The reaction mixture (0.3 ml) contained Tris-HCl, pH 8.0, 15 μmoles; β-mercaptoethanol, 3.6 μmoles; $MgCl_2$, 1.2 μmoles; $MnCl_2$, 0.3 μmole; ATP,GTP,UTP, 100 mμmoles each; ^{3}H-CTP, 50 mμmoles (3000 cpm/mμmole); calf thymus DNA, 20 μg; 7 μg of RNA polymerase and where indicated B44p, 2 μg/ml. A=ATP, G=GTP, U=UTP, C*=^{3}H-CTP and Enz=RNA polymerase.

DNA template to form the DNA-enzyme complex, but it inhibits initiation of RNA synthesis by DNA-dependent RNA polymerase of E. coli. As reported in a previous paper (Mizuno et al., 1967), this antibiotic does not inhibit RNA synthesis by DNA-dependent RNA polymerase of Ehrlich carcinoma cells. This antibiotic has low toxicity to mice. The study of mode of action of the antibiotic B44p presented in this paper suggests that there is a difference

in initiation step of RNA synthesis between bacterial cells and mammalian cells, and it suggests a new basis for chemotherapy against bacterial diseases.

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