ON RNA-POLYMERASES OF LEUKEMIA L1210 ORIGIN AND AN ENZYMATIC METHOD TO SCREEN ANTITUMOR ANTIBIOTICS

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(Received for publication April 5, 1977)

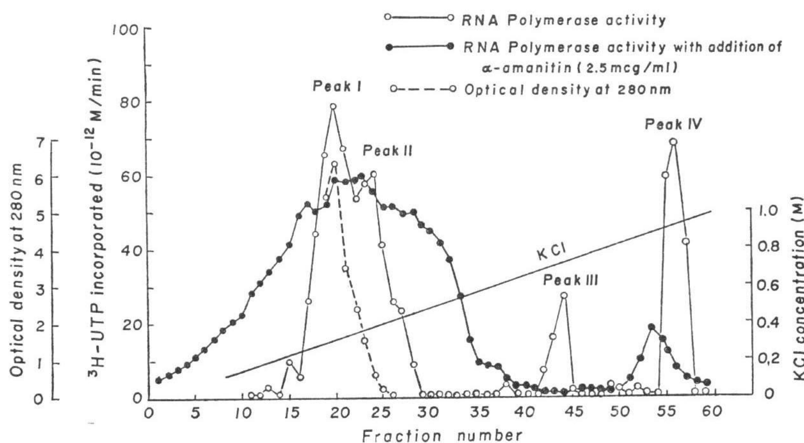
Four DNA-dependent RNA-polymerases were separated from the cell homogenate of mouse Leukemia L1210 cells by DEAE-cellulose column chromatography and tentatively designated as Peaks I, II, III and IV in the elution order. Peak II was inactivated by the addition of α-amanitin and effects of antibiotics and enzymes on the RNA-polymerase activity using Peaks I, II and a mixture of Peaks I and II were examined. The RNA-polymerases were used to screen for enzyme inhibitors produced by microbes. This enzymatic method was successfully proved to select antitumor antibiotics.

The first biochemical method to screen for antitumor antibiotics was reported by NITTA *et al*,[1] and the method was successfully applied to disclose phenomycin[2] as an inhibitor for protein synthesis by a cell-free homogenate of EHRLICH carcinoma cells. Many antitumor antibiotics have been considered to inhibit DNA or RNA synthesis by tumor cells, but none of them shows direct inhibition for RNA-polymerase of mammalian origin. Sarkomycin is quite unique because of its direct inhibition of DNA-polymerase[3]. Nevertheless, rifampicin[4] and streptolydigin[5] are shown as inhibitors of RNA polymerase of microbial origin but not for that of mammalian origin.

DNA-dependent RNA-polymerases of eukaryote origin are more complicated than that of prekaryote origin and three RNA-polymerase activities have been isolated from developing sea urchin and two RNA-polymerase activities have been found in rat liver nuclei by ROEDER and RUTTER in 1969[6,7].

Fig. 1. Elution profile of the RNA-polymerases from a DEAE-cellulose column.

The assay was carried out under the standard condition described in 'Assay method' except 'Addition' was only 50 mM Tris-HCl buffer (pH 7.9)



DNA-dependent RNA-polymerase II from EHRLICH ascites tumor cells was partially purified and characterized by NATORI *et al* in 1973[8,9].

We will report on the isolation of DNA-dependent RNA-polymerases of Leukemia L1210 cells and their application to screen antitumor antibiotics in this paper.

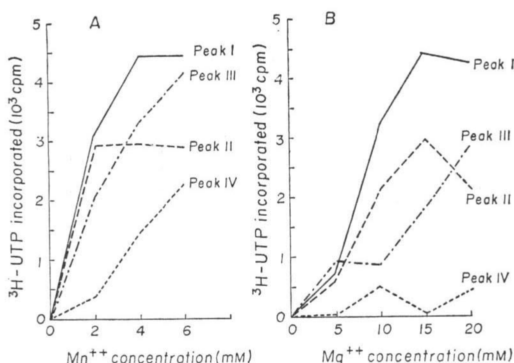
The method described by NATORI, *et al*[8,9] was modified to prepare a supernatant of RNA-polymerase mixture from Leukemia L1210 cells. The filter paper disc method[10~12] was modified to measure the RNA-polymerase activity as described in 'Experimental'. The supernatant derived from about 1×10^9 of the packed cells was partially purified on DEAE-cellulose eluted with a linear gradient concentration of a potassium chloride solution from 0 to 1.0 M. The four peaks of RNA-polymerase activity were respectively eluted at the salt concentrations of 0.32, 0.38, 0.73 and 0.94 M and tentatively designated as Peaks I, II, III and IV in the elution order as shown in Fig. 1. Peak II can be considered to be RNA-polymerase II derived from nucleoplasm, because of its inactivation by the addition of α-amanitin.

Fig. 2. Effect of divalent ions on the RNA-polymerase activity of a mixture of Peaks I and II.

The assay was carried out under the standard condition described in 'Assay method' except Mg^{++} was omitted from 'A-solution' for (A) and Mn^{++} was omitted from 'A-solution' for (B).

'Addition' was only 50 mM of Tris-HCl buffer (pH 7.9).

The activity of each Peak was plotted as zero when the concentrations of both ions were zero.



Effects of Mg^{++} and Mn^{++} ion strength on the RNA-polymerase activity of each Peak are studied in Fig. 2.

Effects of various enzymes, antibiotics and α-amanitin on the RNA-polymerase activity of Peak I, Peak II and a mixture of Peaks I and II are listed respectively in Tables 1, 2 and 3. Ribonuclease A,

Table 1. Percent inhibition by the addition of an enzyme or an antibiotic on the RNA-polymerase activity of a mixture of Peaks I and II.

The assay was carried out under the standard condition described in 'Assay method'.

Enzyme or antibiotic	Concentration (mcg/ml)											
	1,000	500	100	50	10	5	2	1	0.5	0.1	0.05	0
Ribonuclease A			100	100	100			75	18			0
Deoxyribonuclease-1			100	100	95			80	59			0
Pronase			73	60	49			10				0
α-Amanitin							66	70	68	67	36	0
Actinomycin D					100			100	83			0
Chromomycin A_3					100			93	96			0
Pluramycin					100	98		57	46			0
Rifampicin			7		0			0				0
Streptolydigin	52	27	10		0			0				0
Formycin			0		0			0				0
Ilamycin			0		0			0				0
Naramycin			5		0			0				0

Table 2. Percent inhibition by the addition of an enzyme or an antibiotic on the RNA-polymerase activity of Peak I.

The assay was carried out under the standard condition described in 'Assay method' except Peak I was used as 'RNA-polymerase solution'.

Enzyme or antibiotic	Concentration (mcg/ml)							
	20	10	5	1	0.5	0.1	0.05	0
Ribonuclease A		100	95	71	51			0
Deoxyribonuclease-1		100	96	91	54	15		0
α-Amanitin				20	16	8	0	0
Actinomycin D				97	83	55	26	0
Chromomycin-A_3			87	79	48	9		0
Adriamycin	46	44	44	11	14			0

Table 3. Percent inhibition by the addition of an enzyme or an antibiotic on the RNA-polymerase activity of Peak II.

The assay was carried out under the standard condition described in 'Assay method' except Peak II was used as 'RNA-polymerase solution'.

Enzyme or antibiotic	Concentration (mcg/ml)							
	20	10	5	1	0.5	0.1	0.05	0
Ribonuclease A			96	41	38	24	9	0
Deoxyribonuclease-1			74	66	59	10	10	0
α-Amanitin				83	74	63	52	0
Actinomycin D				76	63	33		0
Chromomycin-A_3			87	77	66	55		0
Pluramycin			65	37	24	0		0

deoxyribonuclease-1 and pronase inhibit the RNA-polymerase activities by this method as can be predicted. α-Amanitin inhibited mainly the enzyme activity of Peak II[13,14]. Actinomycin D[15], chromomycin A_3[16], pluramycin[17] and adriamycin[16] showed significant inhibition for the RNA-polymerase activities. The mode of action of the above antibiotics is that they bind to a template DNA and inhibit the DNA-dependent RNA or DNA synthesis. Further, these antibiotics showed also strong inhibition for RNA-polymerase activity using the RNA-polymerase derived from *Escherichia coli* instead of that of Leukemia L1210 origin. Essentially no inhibition for the RNA-polymerase activity was observed by the addition of rifampicin[4] or streptolydigin[5] which show direct inhibition only for the prekaryotic RNA-polymerase but not for the eukaryotic RNA-polymerase. Moreover, negligible inhibition for the enzyme activity was shown by the addition of formycin, ilamycin or naramycin.

Effects of pH, ion strength[18] and EDTA on the RNA-polymerase activities were examined and the results are shown in Table 4. Those

Table 4. Effects of pH of 'Addition', ion strength and EDTA on the RNA-polymerase activity of a mixture of Peaks I and II.

The assay was carried out under the standard conditions described in 'Assay method'.

pH	5.9	6.9	7.9	8.9	9.9
Inhibition (%)	45	35	0	37	74
Effect of ion strength					
Concentration of KCl (mM)	400	200	100	50	0
Inhibition (%)	92	75	25	22	0
Effect of EDTA					
Concentration of EDTA (mM)	20	10	5	2.5	0
Inhibition (%)	75	5	0	0	0

effects can not be neglected in applying the enzymatic method to screen antitumor antibiotics.

The mixture of Peaks I and II was conveniently used to screen the enzyme inhibitors produced by microbes and two microbes out of 150 strains showed significant inhibition. Those two microbes did not show any antimicrobial activity against bacteria, yeasts or fungi. The active component of one strain was found to be a basic polypeptide which could not be dialyzed. The other strain produced a metabolite which inhibited the RNA-polymerase activity of Leukemia L1210 origin but not the RNA-polymerase of *E. coli* origin. Isolation and purification of the latter metabolite are now under investigation and will be reported in the near future.

Experimental

Materials used in this experiments were as follows:

Hemolysis buffer: 0.747% (w/v) NH_4Cl and 17 mM Tris-HCl (pH 7.5).

HANKS' solution: 0.8% (w/v) NaCl, 0.04% (w/v) KCl, 0.035% (w/v) $NaHCO_3$, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 0.014% (w/v) $CaCl_2$, 0.006% (w/v) KH_2PO_4 and 0.006% (w/v) Na_2HPO_4.

Buffer A: 50 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.09 mM mercaptoethanol and 25% (v/v) glycerin.

Buffer Ad: 0.5 mM Dithiothreitol was added to Buffer A.

ATP, GTP, CTP, UTP, calf thymus DNA and α-amanitin were purchased from Sigma Chem. Co.

^{3}H-UTP (tetra sodium salt, 25.2 Ci/mM) was purchased from New England Nuclear.

BDF_1 strain mice were purchased from the Shizuoka Animal Farm Coop.

Assay Method

The standard assay medium (total volume of 250 μl) contained: (1) 50 μ of XTP solution composed of 0.1 μM each ATP, GTP and CTP, 0.01 μM UTP and 1 μCi ^{3}H-UTP; (2) 25 μl of A-solution composed of 3.0 μM Tris-HCl of pH 7.9, 0.015 μM EDTA, 12.5 μM $(NH_4)_2SO_4$, 2,5 μM $MgCl_2$, 0.5 μM $MnCl_2$ and 0.75 μM mercaptoethanol; (3) 25 μl of native calf thymus DNA solution (1,000 μg/ml); (4) 50 μl of Addition (a test sample dissolved in 50 mM Tris-HCl of pH 7.9) and (5) 100 μl of RNA polymerase solution.

After incubation for 1 hour at 37°C, test samples were chilled in ice. Then, 100 μl of each sample was applied to two filter papers (Toyo filter paper, No. 52, 1.8 × 1.8 cm), immersed in 500 ml of cold 10% TCA solution containing 0.01 M of sodium pyrophosphate for 20 minutes, washed three times with 500 ml of cold 5% TCA containing 0.01 M sodium pyrophosphate for 20 minutes and washed three times with 200 ml of EtOH and dried. The radioactivity on the filter paper was counted using a Packard scintillation counter.

Preparation of 'RNA-Polymerase Solution'

Leukemia L1210 cells (4×10^5/mouse) were injected into the abdominal cavity of a BDF_1 mouse of 4 ~ 7 weeks old. The cells were harvested after 7 days, washed with physiological saline, the hemolysis buffer, HANKS' solution, buffer-A and finally with buffer-Ad and stored at −30°C. About 1×10^9 cells harvested from 20 ~ 25 mice (about 10 ml, packed volume) were suspended in two volumes of buffer-Ad, disrupted in a glass homogenizer by 70 strokes with a Teflon piston and 0.077 ml/ml of 4 M $(NH_4)_2SO_4$ was added to the homogenate. The viscous solution was sonicated 15 ~ 16 times for 15 seconds with 1-minute intervals using the Ohtake sonicator at nearly maximum power until the viscosity was decreased. Then, 2.5 volumes of buffer-Ad were added to the sonicated solution and centrifuged for 1 hour at 100,200 × *g* (Hitachi Ultracentrifuge 65P, rotor RP65T) to prepare 'RNA polymerase supernatant'. The supernatant was filtered on cotton and diluted by adding an equal volume of buffer-Ad. This diluted enzyme solution was applied to a column of DEAE-cellulose (5 × 1.7 cm diam., equilibrated with buffer-Ad) and washed with 100 ml of buffer-Ad. The mixture of Peaks I and II was eluted with 10 ml of 0.6 M KCl in buffer-Ad and each 10 ml of buffer-Ad and glycerin were added to the eluate. 'RNA-polymerase solution', thus obtained, was stored at −70°C. All operations were carried out at 0 ~ 5°C.

Fractionation of RNA-Polymerases on DEAE-Cellulose

The packed cells of Leukemia L1210 (1×10^9) harvested from 26 BDF_1 mice was treated as before mentioned to prepare 'RNA-polymerase supernatant'. The supernatant was applied to a column of DEAE-cellulose (4.5×1.7 cm diameter, equilibrated with buffer-Ad). The column was eluated with a linear gradient concentration of KCl from 0 to 1.0 M after washing with 100 ml of buffer-Ad. The eluate was fractionated in each 2.1-ml fractions and 1 ml of glycerin was added to each fraction. The assay of the RNA-polymerase activity was carried out under the standard condition described in 'Assay method' except 'Addition' was only 50 mM Tris-HCl buffer.

Screening for RNA Polymerase Inhibitors Produced by Microbes

A culture filtrate of a microbe was diluted to make 10-fold diluted solution with 50 mM Tris-HCl buffer (pH 7.9) after heating at 80°C for 5 minutes. The diluted filtrate was used as 'Addition' and screened for the inhibitor under the standard condition. An inactivated enzyme solution was used as 'RNA polymerase solution' as the positive control, while 50 mM Tris-HCl buffer (pH 7.9) was used as 'Addition' instead of the negative control.

Acknowledgment

The authors wish to express their appreciation to Prof. H. UMEZAWA and Prof. M. HORI, Institute of Microbial Chemistry, for their helpful advices. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Welfare, Japan, to which we are deeply indebted.

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