INHIBITION OF MAMMALIAN RNA POLYMERASES BY A PROTEIN FACTOR FROM EHRLICH ASCITES TUMOR CELLS

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1. Introduction

The study of the mechanisms regulating RNA synthesis in eukaryotes has led to the isolation of stimulating [1–5] and repressing factors [6–8] of RNA polymerases in vitro. Some of these factors have a narrow specificity: enzyme or template specificity. From Ehrlich ascites tumor cells, Natori et al. have isolated a factor repressing RNA polymerase II [6,7]. It is a ribonucleoprotein which inhibits the transcription of native DNA by homologous RNA polymerase II. A non histone protein isolated from Ehrlich cell chromatin inhibits the transcription of native DNA by eukaryotic RNA polymerase II. It inhibits preferentially the transcription of homologous DNA [8].

In this paper, we report the isolation from nuclei of Ehrlich ascites tumor cells of a protein factor which inhibits the transcription of native ascites DNA by mammalian RNA polymerase A or I (located in the nucleolus) and RNA polymerase B or II (located in the nucleoplasm).

2. Materials and methods

Materials and their sources were described previously [9].

2.1. *Polymerase assay*

The assay mixture contains in 0.16 ml total volume, 62.5 mM of Tris-HCl pH 7.9, 3 mM $MnCl_2$, 18 μg DNA (350 mM), ammonium sulfate 75 mM, 0.25 mM ATP, GTP and CTP, 0.03 mM [^{3}H]UTP (20–100 counts × min^{-1} × $pmol^{-1}$). Enzyme is added in the following buffer: 50 mM Tris–HCl pH 7.9, 10 mM thioglycerol, 0.1 mM EDTA, 0.1 mM dithio-1,4-erythritol, glycerol 30%, v/v. Assays were incubated for 20 min at 37°C and collected on a Whatman paper no 1 (4 × 4 cm). Whatman papers were washed 3 times with cold 5% perchloric acid, 0.1 M sodium pyrophosphate, for a few hours and 3 times with an ether–ethanol mixture (1/1). After drying for 10 min at 60°C, papers were counted with a Beckman scintillation counter in vials containing 5 ml of scintillator (PPO 0.6%, w/v; POPOP 0.03%, w/v in toluene). Values of the blank assays (not incubated) were subtracted. A unit of enzyme activity is defined as the amount of enzyme required to incorporate 1 pmol UTP/min of incubation under the conditions described above. All assays were made in duplicate.

2.2. *Assay of inhibition or stimulation of RNA synthesis*

Fractions to be tested, 10–100 μl, were added to the assay mixture before incubation with enzymes.

3. Results and discussion

During the last step of the purification of ascites RNA polymerases [9], the protein fractions eluted from the DEAE cellulose column were systematically tested for their repressing or stimulating activities towards ascites RNA polymerases A and B. No stimulating factor was detected. The material excluded

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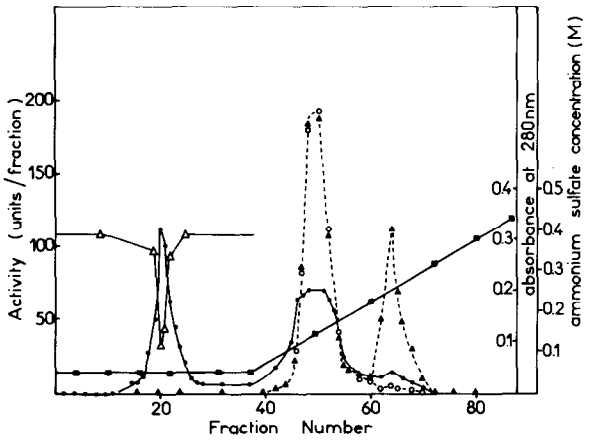

Fig.1. DEAE cellulose chromatography of inhibitory factor and of RNA polymerase A and B activities. DEAE cellulose column was equilibrated with the following buffer : 50 mM Tris-HCl, pH 7.9, 10 mM thioglycerol, 0.1 mM EDTA, 0.1 mM dithio-1,4-erythritol, 0.05 M ammonium sulfate, glycerol 30%, v/v. 30 ml corresponding to 1–2 mg/ml of protein were loaded on the column (1.5 × 30 cm) at a flow rate of 11–15 ml/h and allowed to stand for 1 h. The resin was washed with 50–100 ml of the buffer. Elution was performed with a linear 0.05–0.6 M ammonium sulfate gradient in 300 ml of the buffer at a flow rate of 25 ml/h. Fractions of 4 ml were collected and kept at −70°C. Enzyme activity is detected on aliquots (60 μl) incubated with 1.25 μg of α-amanitin (inhibitor of RNA polymerase B) or without α-amanitin. (●——●) A_{280}; (■——■) ammonium sulfate gradient; (○——○) activity of RNA polymerase in the presence of α-amanitin; (▲——▲) activity of RNA polymerase in the absence of α-amanitin; (△——△) inhibitor activity (30 μl of inhibitor A_{280} = 0.4, with Ehrlich ascites RNA polymerase A and ascites DNA).

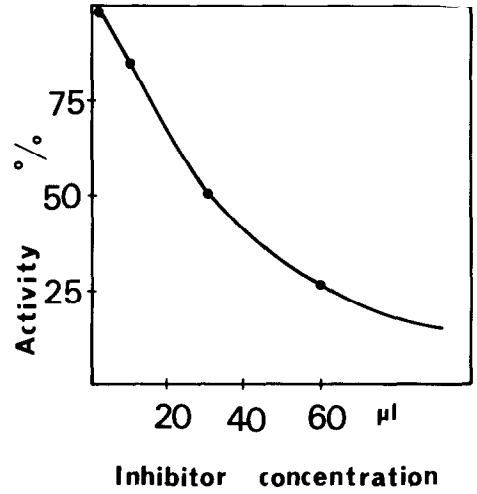

Fig.2. Inhibition of RNA polymerase B from Ehrlich ascites tumor cells by the inhibitory factor with native ascites DNA template. Ammonium sulfate concentration is maintained constant by addition of aliquots of 0.05 M ammonium sulfate. Concentration of the solution containing the inhibitory factor : A_{280} = 0.4.

on DEAE cellulose column strongly inhibits RNA polymerases A and B (fig.1). The inhibitory effect increases with factor concentration (fig.2). The activity of this factor has been tested with RNA polymerases A and B from Ehrlich ascites tumor cells and from calf thymus, using two different mammalian DNA templates: one is the homologous nuclei ascites DNA, which is composed of 90% of a main component (42% GC base pairs) and 10% of a light satellite (31.6% GC base pairs); the other one is the calf thymus DNA whose base composition is not very different (44% GC base pairs). The calf genome contains at least 4 satellite DNAs which represent 15% of the total DNA. The kinetics of reassociation of ascites tumor total DNA [10] and calf thymus DNA [11] show that both DNAs have the same proportion of highly reiterated sequences. The molecular weight of nuclei ascites DNA varies, according to the different preparations, between 8.2 to 15.7 × 10^6 and in the case of calf thymus DNA between 6 and 8 × 10^6. No single strand breaks can be detected either in ascites or in calf thymus DNA, by the technic of Sicard et al. [12]. Therefore, it seems relevant to compare the action of the inhibitory factor towards these two DNAs whose physico-chemical properties are similar. The results are summarized in table 1. This factor inhibits RNA synthesis with the four enzymes tested but only with ascites DNA template. The inhibition cannot be attributed to a degradation of newly synthesized RNA since the transcription of calf thymus DNA is not inhibited.

The factor activity is destroyed by digestion (5 min, 37°C) with different amounts of trypsin. After cooling at 4°C, trypsin action is stopped by addition of soy bean trypsin inhibitor. The decrease of repressing activity is related to the quantity of trypsin added. The inhibitory activity is also suppressed by treatment with an equal volume of chloro-

Table 1
Effect of the inhibitory factor on RNA synthesis by ascites or calf thymus RNA polymerases with ascites or calf thymus DNA templates

RNA polymerase	DNA	
	Calf thymus	Ascites
Ascites A	104	30
Ascites B	106	53
Calf thymus A	90	58
Calf thymus B	100	46

% activity has been calculated with reference to DNA without inhibitor. Inhibitor concentration: 30 μl (A_{280} = 0.4) in the assay medium described in the text. 30 μl of the buffer containing 0.05 M ammonium sulfate are added in control assays

form–isoamyl alcohol (9:1) which is a denaturing agent of proteins.

Since some repressing factors are nucleoproteins [7,13], the presence of nucleic acid has been checked in this inhibitory factor. Incubation with endonuclease from *Staphylococcus aureus* is without action on the inhibitory activity. Furthermore no inorganic phosphate has been detected after complete hydrolysis of the molecule. Therefore, it can be assumed that the inhibitory factor does not contain nucleic acid.

In order to prove that the inhibitory activity is not due to a contamination by a nuclease destroying the DNA template, ascites DNA was incubated with the inhibitor and thereafter treated with an equal volume of chloroform–isoamyl alcohol (9:1). The DNA which is contained in the aqueous phase is precipitated by 2 volumes of cold ethanol and dialysed against the appropriate solvent. The yield of DNA is 100%. The physicochemical properties of the DNA are not modified (table 2). The repressing activity can hardly be attributed to the very slight number of single strand breaks. This DNA is normally transcribed by RNA polymerases and the transcription can be inhibited by a subsequent addition of the inhibitor.

In conclusion, it can be assumed that the inhibitory factor isolated from purified nuclei of Ehrlich ascites tumor cells inhibits selectively the transcription of homologous DNA. This factor is not enzyme specific since it inhibits RNA synthesis not only by RNA polymerase A and B from Ehrlich cells but also by RNA polymerases A and B from calf thymus. It does not interfere with another inhibitor of transcription, the antibiotic antimitotic daunorubicin. This drug interacts with DNA without base specificity and inhibits enzymic reactions (DNAases, RNA polymerases) by formation of an inactive complex DNA-enzyme-daunorubicin [9,13,14]. The presence of the protein inhibitor is without influence on the kinetics of inhibition of RNA synthesis by daunorubicin (fig.3).

This factor is not a nucleoprotein, therefore it is different from the ribonucleoprotein factor characterized in [7]. As it inhibits the transcription by RNA polymerase A as well as the transcription by RNA polymerase B, it is also different from the non histone chromosomal protein factor

Table 2
Some physicochemical properties of ascites DNA before and after incubation with the inhibitory factor

Ascites DNA	T_m (0.1 SSC)	S_N	S_A	M_N 10^{-6}	M_A 10^{-6}	Single strand breaks
Native	71.5	27.3	29.4	15.7	7.41	0
After incubation with the inhibitory factor	70.5	26.8	24.0	14.9	4.41	0.68

S_N and S_A; sedimentation constant in neutral or alkaline medium. M_N (molecular weight in neutral medium, NaCl M) and M_A (molecular weight in alkaline medium, 0.9 M NaCl–0.1 M NaOH) are estimated according to Studier [13]. Single strand breaks have been calculated according to Sicard et al. [12]

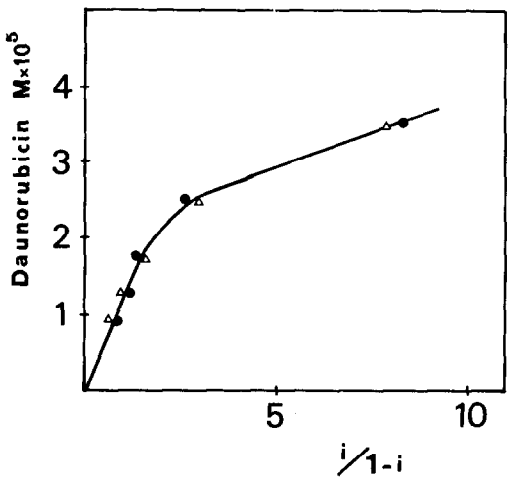

Fig.3. Inhibition of RNA polymerase by daunorubicin in the absence or in the presence of the inhibitory factor. Results are expressed according to Reiner [13]. The ordinate shows daunorubicin concentration and the abcissa i/1−i where i = (rate without daunorubicin − rate with daunorubicin)/(rate without daunorubicin). Incubation medium: $MnCl_2$, 2 mM; $(NH_4)_2SO_4$, 94 mM; ascites DNA concentration 1.2×10^{-4} M, 50 µl RNA polymerase B from Ehrlich ascites cells. (△——△) Without protein inhibitor; (●——●) in the presence of the protein inhibitor (30 µl, $A_{280} = 0.4$) added in the incubation medium together with daunorubicin and before RNA polymerase.

which is specific for eukaryotic RNA polymerase II and binds specifically to the reiterated sequences of the DNA [8]. Attempts to define the role of these repressing or stimulating factors can lead to a better knowledge of the mechanism of transcription by RNA polymerases not only in vitro, but moreover in vivo.

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References

[1] Natori, S., Takeuchi, K. and Mizuno, D. (1973) J. Biochem. 73, 879–888.
[2] Froehner, S. C. and Bonner, J. (1973) 12, 3064–3071.
[3] Stein, H. and Hausen, P. (1970) Eur. J. Biochem. 14, 270–277.
[4] Nagamine, Y., Natori, S. and Mizuno, D. (1976) FEBS Lett. 67, 198–201.
[5] Kuroiwa, A., Sekimizu, K., Ueno, K., Mizuno, D. and Natori, S. (1977) FEBS Lett. 75, 183–186.
[6] Natori, S., Takeuchi, K. and Mizuno, D. (1974) J. Biochem. 76, 263–270.
[7] Natori, S., Takeuchi, K. and Mizuno, D. (1975) J. Biochem. 77, 1319–1323.
[8] Kostraba, N. C., Newman, R. S. and Wang, T. Y. (1977) Arch. Biochem. Biophys. 179, 100–105.
[9] Barthelemy-Clavey, V., Molinier, C., Aubel-Sadron, G. and Maral, R. (1976) Eur. J. Biochem. 69, 23–33.
[10] Markov, G. G. and Ivanov, I. G. (1977) Neoplasma 24, 497–506.
[11] Britten, R. and Kohne, D. E. (1968) Science 161, 529–540.
[12] Sicard, P. J., Obrenovitch, A. and Aubel-Sadron, G. (1972) Biochim. Biophys. Acta 268, 468–479.
[13] Steward, L. E. and Krueger, R. C. (1976) Biochim. Biophys. Acta 425, 322–333.
[14] Studier, F. W. (1965) J. Mol. Biol. 11, 373–390.
[15] Barthelemy-Clavey, V., Maurizot, J. C. and Sicard, P. J. (1973) Biochimie 55, 859–868.
[16] Barthelemy-Clavey, V., Serros, G. and Aubel-Sadron, G. (1975) Mol. Pharmacol. 11, 640–646.
[17] Reiner, J. M. (1969) Behaviour of enzymes systems, pp. 192–199, Burgess, Minneapolis.