NUCLEAR DNA-DEPENDENT RNA POLYMERASES OF EHRLICH ASCITES TUMOR CELLS: TWO DISCRETE α-AMANITIN-SENSITIVE FORMS

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SUMMARY

DNA-dependent RNA polymerase was solubilized from nuclei of ascites tumor cells by the standard techniques of ultrasonic treatment in 0.3 M ammonium sulfate, salt fractionation, and dialysis. Three discrete forms of RNA polymerase (I, II, III) were separated on DEAE-Sephadex A-25. Forms II and III were inhibited by α-amanitin, but no form was sensitive to rifampicin. Each form was more active with Mn^{++} than with Mg^{++} ions, more active with denatured than with native calf thymus DNA, and differed from the others with respect to optimal concentrations of $(NH_4)_2SO_4$, Mn^{++} ions and DNA.

INTRODUCTION

Five multiple DNA-dependent RNA polymerases have been detected in nuclei of eukaryotic cells (1-5). According to the nomenclature of Kedinger et al. (4) these are AI, AII, AIII, BI and BII, the A forms being sensitive, and the B ones insensitive, to inhibition by α-amanitin. Forms AI and B, which constitute the major proportion of the RNA polymerase activity in the nucleus (6,7), correspond to forms I and II of Rutter and associates (1), and form AIII is the same as form III of the Rutter group (1). Forms AI and AII are found in the nucleolus (8,9), whereas the others are located in the nucleoplasm (1,8,9).

It has been known for some time that neoplasia alters RNA polymerase activity (10). Thus, a study was begun of the number, structure and function of RNA polymerases in the nuclei of Ehrlich ascites carcinoma cells. While this study was in progress, Chesterton et al. (7) reported that, relative to the DNA content of the tissues, the activities of forms AI, AII and B of the enzyme were somewhat higher in a minimal-deviation rat hepatoma cell line than in rat liver, but detected no new RNA polymerases in liver or hepatoma cells. Furth and Austin (11) have

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isolated multiple RNA polymerases from bovine lymphosarcoma but the relationship of their enzymes to those described by others (1-5) is not clear. The results of the studies which we describe here suggest that nuclei of Ehrlich ascites cells contain three forms of RNA polymerase, one of which is different from those found previously (1-5) in eukaryotic cells. The characteristics of the partially purified RNA polymerase (about 5-fold) are described elsewhere (12,13).

MATERIALS AND METHODS

The Ehrlich ascites carcinoma was propagated in male Swiss mice by weekly, serial, intraperitoneal transfer of 2-6 x 10^6 cells. Cells were harvested from mice inoculated 7-9 days previously, pelleted by centrifugation at 4° C, and washed once with hypotonic salt solution to lyse erthrocytes (14). The remaining cells were washed twice with Hanks' balanced salt solution (15), and nuclei were isolated from them by treatment with 1% saponin essentially according to the method of Read and Mauritzen (16), who have shown that this treatment does not adversely affect RNA polymerase activity. The nuclei were washed with 0.25 M sucrose-10 mM potassium phosphate buffer (pH 7.0)-4 mM EDTA until most of the cytoplasm was removed, resuspended in 6 volumes of 2.4 M sucrose-5 mM $MgCl_2$, and centrifuged for 1 hour at 30,000 x g (16,17). Purified nuclei were stored at -70° C in the presence of 5 mM dithiothreitol (DTT) (18) for up to two weeks without loss of activity.

RNA polymerase was solubilized from nuclei essentially according to the procedure of Roeder and Rutter (8), but with 2 minutes of sonication in 0.3 M $(NH_4)_2SO_4$ (13). The protein precipitated from the 105,000 x g supernatant fraction (F2) of the sonicate (F1) by $(NH_4)_2SO_4$ (0.42 g/ml) was dissolved in TGMED buffer (0.05 M Tris, pH 7.9-25% glycerol-5 mM $MgCl_2$-0.1 M EDTA-0.5 mM DTT) to form fraction F3 which was dialyzed for 4-8 hours at 4° C vs. TGMED buffer (pH 7.9) containing 0.05 M $(NH_4)_2SO_4$. The diffusate was centrifuged at 105,000 x g for 1 hour at 4° C and the resultant supernatant fraction (F4) was used for initial studies (12,13) of the RNA polymerase reaction and for the chromatographic separation of RNA polymerase forms. F4 fractions were stored at -70° C for up to 2 weeks with little or no loss of activity. The protein content of the

of the fractions was determined by the method of Lowry et al. (19). DNase and RNase activities of the F4 fraction were estimated by the methods of Kunitz (20) and of Kalnitsky et al. (21), respectively.

RNA polymerases of fraction F4 were separated by chromatography on DEAE-Sephadex A-25 (18). The enzymes were eluted by a stepwise gradient of $(NH_4)_2SO_4$ (11) as described in the legend to Fig. 1. The protein content of each fraction (1 ml) was estimated from the ratio of the absorption at 280 nm to that at 260 nm, and RNA polymerase was determined as described below.

RNA polymerase was assayed by the incorporation of radioactivity from ^{3}H-UTP into acid-insoluble RNA. A unit of activity is defined as the amount of enzyme which catalyzes the incorporation of 1 pmole of ^{3}H-UMP into acid-insoluble product in 1 minute at 37° C. The assay medium was the standard one of Roeder and Rutter (22). The reaction medium (0.25 ml) contained 0.1 ml of enzyme (F4 or chromatographic fraction) in TGMED (pH 7.9)-0.05 M $(NH_4)_2SO_4$. The final concentrations of components were: native calf thymus DNA, 160 µg/ml; pyruvate kinase (Calbiochem), 20 µg (6.8 I.U.) per ml; phosphoenolpyruvate (Sigma), 4 mM; 2-mercaptoethanol, 1.6 mM; NaF, 6 mM; KCl, 8 mM; $MnCl_2$, 1.6 mM; ATP, CTP and GTP, 0.6 mM each; UTP, 0.1 mM; ^{3}H-UTP (Amersham/Searle), 0.004 mM (4 µCi/ml); $(NH_4)_2SO_4$, 0.05 M; Tris-HCl buffer (pH 7.9), 76 mM; $MgCl_2$, 2mM; EDTA, 0.05 mM; DTT, 0.2 mM; and glycerol, 10%. After an incubation period of 10 min at 37° C, the reaction tubes were placed in ice, and 1 ml of 0.1 M $Na_4P_2O_7$ containing 2 mg each of yeast RNA and bovine serum albumin, and 5 mM UTP, was added immediately to each, followed sequentially by 1 ml each of 5% sodium dodecyl sulfate and 20% CCl_3COOH (TCA)-0.04 M $Na_4P_2O_7$. Precipitates were collected on Whatman glass fiber filters (GF/C) which were washed 8 times with 5-ml portions of 5% TCA-0.04 M $Na_4P_2O_7$ and placed in sciltillation vials. The precipitates were solubilized in NCS solubilizer diluted 1:3 with toluene scintillator (0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis-(5-phenyloxazolyl-2)benzene in toluene) (1 ml), toluene scintillator (5 ml) was added, and radioactivity was was determined in a Nuclear-Chicago Unilux I spectrometer. Activity was proportional to enzyme reaction time and enzyme amount for fraction F4 (13). All tests were done in duplicate and no-enzyme controls were subtracted.

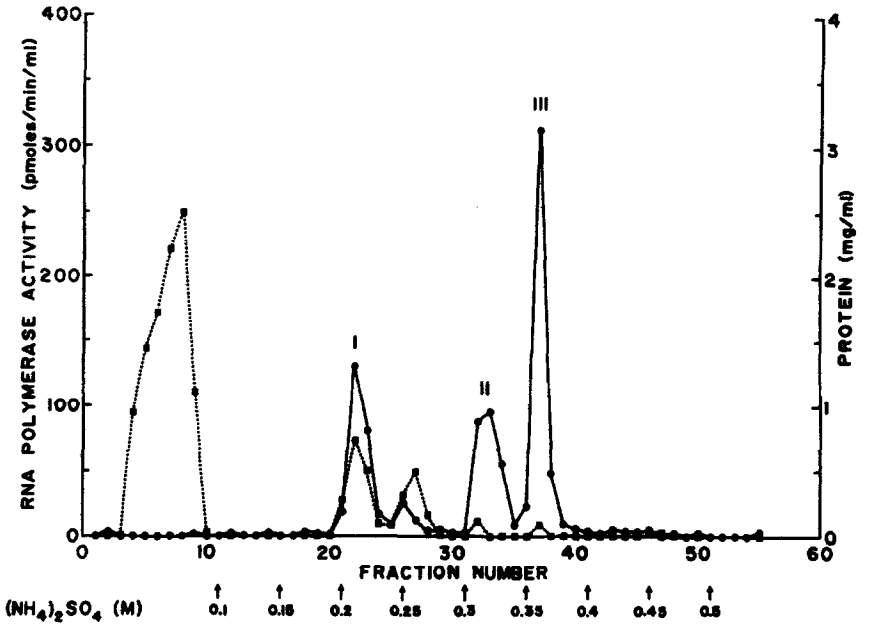

Fig. 1. DEAE-Sephadex chromatography of RNA polymerase (fraction F4). Fraction F4 enzyme (12.4 mg protein in 5 ml) was layed over a 0.9 x 12 cm DEAE-Sephadex A-25 column, washed with 5 ml of TGMED buffer (pH 7.9)-0.05 M $(NH_4)_2SO_4$, and eluted with a step-wise gradient of $(NH_4)_2SO_4$ in TGMED buffer. For this the column was washed successively with 5 ml each of TGMED buffer (pH 7.9) containing 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 M $(NH_4)_2SO_4$, beginning at the fraction indicated. Fractions of 1.0 ml were collected and assayed (0.1 ml of each fraction) as described in the text. Protein was estimated by the ratio of optical density at 280 nm to that at 260 nm. Activity, ●——●; protein, ■·····■.

The RNA polymerase activity of fraction F4 is dependent upon the presence of DNA, the four ribonucleoside triphosphates and Mn^{++} ions, and is inhibited by actinomycin D and chromoycin A3, but not by rifamycin or rifampicin (13). The enzyme purification is about 5-fold from nuclei, and RNase and DNase activities are barely detectable by standard methods (13). The best commercial grades of chemicals were used. Bovine serum albumin (fraction V), and rifampicin were obtained from "Schwarz/Mann"; yeast total RNA from "Sigma" and calf thymus DNA from "Worthington".

RESULTS AND DISCUSSION

Three major, discrete enzyme activities were obtained by chromatography of the F4 fraction on DEAE-Sephadex A-25 (Fig.1). Tentatively, these have been designated I, II and III, primarily

Table I. Characteristics of multiple nuclear RNA polymerases from Ehrlich ascites tumor cells

Condition	RNA polymerase I	II	III
Elution from DEAE-Sephadex by $(NH_4)_2SO_4$ (M)	0.2	0.3	0.35
Distribution of activity on chromatogram (mean of 7) (%)	25.7	32.7	41.6
Optimal Mn^{++} concentration (mM)	2.4	1.6	2.0
Optimal Mg^{++} concentration (mM)	4-8	4-8	4-8
Ratio of activities at optimal ion concentrations (Mn^{++}/Mg^{++})	2.6	1.7	6.0
Optimal concentration of native calf thymus DNA (µg/ml)	16	80	8
Ratio of activities at optimal calf thymus DNA concentrations (denatured-/native)	2.1	2.9	3.7
Optimal $(NH_4)_2SO_4$ concentration in assay (M)	0.08	0.09	0.035-0.10
Inhibition by α-amanitin (3µg/ml) (%)	0	75	84
(9 µg/ml) (%)	0	100	97
Inhibition by rifampicin (6 µg/ml) (%)	24	0	8
(59 µg/ml) (%)	29	2	3

RNA polymerase was assayed as described in the text. An amount of RNA polymerase containing 10 or more units of activity was used for each reaction. Inhibitors were added to reaction media just prior to the enzyme, which was added immediately prior to incubation. Enzymes dialyzed for 8 hours at 4° C versus TGMED buffer (pH 7.9)-0.05 M $(NH_4)_2SO_4$ lacking Mg^{++} ions or $(NH_4)_2SO_4$, were used to determine optimal Mg^{++} or $(NH_4)_2SO_4$ concentrations.

because their chromatographic and enzymatic activities resemble those of the rat liver forms (22). The chromatographic separation is highly reproducible and results in a purification of about 7-, 10- and 200-fold for forms I, II and III, respectively, from the F4 fraction. The RNA polymerase of the particular F4 preparation which was chromatographed had been purified approximately 4-fold from nuclei. Form III was always the major RNA

polymerase, accounting for 42% of the activity. Table I summarizes the properties of the three enzyme forms derived from several chromatographic separations. The properties of forms I and II are similar to those of forms I and II from other eukaryotic cells and tissues (2,7,22,23) but, whereas enzyme III from rat liver is insensitive to α-aminitin, the form which we have designated III definitely is sensitive. On the basis of sensitivity to α-amanitin the Ehrlich actites form III enzyme (as well as form II) is a B form (4,5), but, as shown above, most of its properties are quite different from those of form II. Inhibition by α-amanitin, but not by rifampicin, shows that form III is not of mitochondrial origin (24).

Further purification of form III on DEAE-cellulose and in glycerol gradients, together with structural analyses, must be carried out in order to ascertain whether form III is an entirely new B form of RNA polymerase or an artifact of chromatography. It may be useful for comparative studies of RNA polymerase structure, and will be of great interest if it is unique to neoplastic cells.

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