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Abstract

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KEYWORDS: RNA polymerase, Pous sarcoma virus, mouse ascites sarcoma

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CHARACTERIZATION OF RNA POLYMERASES FROM ROUS SARCOMA VIRUS-INDUCED MOUSE ASCITES SARCOMA CELLS*

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Abstract. RNA polymerase was extracted from the Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV)-induced C3H/He mouse ascites sarcoma cells (SR-C3H). RNA polymerase was separated into RNA polymerases I and II by DEAE-Sephadex chromatography. RNA polymerase I was separated into Ia and Ib fractions by phospho-cellulose chromatography. In SR-C3H cells RNA polymerase Ib was the main component of RNA polymerase I. At 0.05-0.1 M ammonium sulphate RNA polymerase I transcribed native DNA most actively, and RNA polymerase II transcribed denatured DNA most actively. Partial digestion of DNA by DNase I enhanced RNA synthesis by RNA polymerases I and II. At ionic strength over 0.2 M ammonium sulphate, the initiation reaction of RNA polymerases I and II was inhibited. The initiation complexes of RNA polymerases I and II with native DNA were more stable against high salt concentration than with denatured DNA.

Key words : RNA polymerase, Rous sarcoma virus, mouse ascites sarcoma

Mammalian cells can be transformed by the Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV) (17, 18, 25). RSV-transformed mammalian cells have complete viral genomes though the genetic expression of the virus is restricted. The viral genome in host cells is transcribed by host DNA dependent RNA polymerases (E. C. 2. 7. 7. 6) (6), and the process may be controlled by viral and cellular interaction. RNA polymerases from mammalian cells can be resolved into forms I, II, and III by DEAE-Sephadex (or cellulose) chromatography (1, 2, 14). Form I is located in the nucleolus (15) and is responsible for ribosomal RNA synthesis (26). RNA polymerases II and III are located in the nucleoplasm (15) and responsible for transcription of non-ribosomal genes (21, 22, 26). To

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investigate the specific role of RNA polymerase in the expression of viral genomes, we isolated these enzymes from SR-RSV transformed mouse ascites cells and investigated the properties of the enzymatic reaction and the template requirements.

MATERIALS AND METHODS

SR-RSV-induced mouse ascites sarcoma established by Yamamoto and Takeuchi (23) were injected intraperitoneally to C3H/He mice. The cells were harvested 7 days after inoculation. [^{3}H]-UTP was purchased from the Radiochemical Center Ltd., England; dithiothreitol and α-amanitin were from Calbiochem, Calif.; unlabelled ribonucleoside triphosphates were from Boehringer, Manheim Germany; and calf thymus DNA (Type I) was from Sigma Chemical Co., St. Louis. DNase I was obtained from Worthington Biochemical Co., Freehold, N. J.

Preparation of nuclei. The ascites cells (5–15 g) were washed in buffer A (0.25 M sucrose; 10 mM Tris-HCl, pH 7.9; 3 mM $MgCl_2$; 5 mM 2-mercaptoethanol) to eliminate red blood cells. The cells were suspended in 10 volumes of buffer A, filtered through four layers of Teflon cloth, and homogenized by an Emanuel-Chaikoff homogenizer (17). The homogenate was centrifuged at 170×g for 5 min. The crude nuclear pellet was suspended in 2.5 M sucrose containing 10 mM Tris-HCl (pH 7.9); 3 mM $MgCl_2$; and 5 mM 2-mercaptoethanol, and the sucrose concentration was adjusted to 2 M by dilution with buffer A. This was centrifuged for 1 h at 45,000×g (at 4°). The white nuclear pellet was suspended in 1 M sucrose containg 50 mM Tris-HCl, (pH 7.9); 5 mM $MgCl_2$; and 0.5 mM dithiothreitol (1 ml/g of tissue).

Preparation of RNA polymerase. RNA polymerase was extacted by the procedure of Roeder, Blatti and Rutter (20). Saturated ammonium sulphate (4 M, pH 7.9) was added to the nuclear suspension to bring the concentration to 0.3 M, and the suspension was sonicated with 4–6 bursts of 15 seconds duration at 0°C. After stirring for 1 h, the suspension was mixed with two volumes of buffer B (25% v/v glycerol; 50 mM Tris-HCl. (pH 7.9); 5 mM $MgCl_2$; 0.1 mM EDTA; 0.1 mM dithiothreitol) and centrifuged for 30 min at 105,000×g. The supernatant was diluted to 0.033 M $(NH_4)_2SO_4$ with buffer B and centrifuged again at 105,000 ×g for 30 min. The supernatant fraction was immediately applied to a DEAE-Sephadex A-25 column (1.5×7.5 cm) which was previously equilibrated with buffer B containing 0.03 M $(NH_4)_2SO_4$. After washing with this buffer the column was then eluted with five bed-volumes of a linear gradient of 0.03–0.6 M $(NH_4)_2SO_4$ in buffer B. Fractions of 1.2 ml were collected and the activities of RNA polymerase were immediately assayed.

Phosphocellulose chromatography of RNA polymerase I from SR-C3H cells was carried out as follows: SR-C3H cells (52 g) were suspended in 150 ml of 5% v/v glycerol containing 0.05 M Tris-HCl, (pH 7.9); 0.1 mM EDTA; and 10 mM 2-mercaptoethanol and homogenized with a Waring blender at maximal speed for 4 min. Ammonium sulphate (4 M, pH 7.9) was added to the homogenate to a

concentration of 0 3 M. Aliquots of 30 ml were sonicated with 8–12 bursts of 15 seconds duration at 0° and diluted with buffer C (25% v/v glycerol ; 0.05 M Tris-HCl, (pH 7.9) ; 0.1 mM EDTA ; 10 mM 2-mercaptoethanol) and centrifuged at 105,000×g for 1 h. Solid ammonium sulphate was added to the supernatant to 40% of saturation and stirred for 1 h. The precipitate was collected by centrifugation at 45,000×g for 20 min and dissolved in buffer C. This was diluted with the same buffer until the concentration of $(NH_4)_2SO_4$ was 0.03 M. DEAE-cellulose (200 g wet weight) equilibrated in 0.03 M $(NH_4)_2SO_4$ in buffer C was added to this solution and stirred for 1 h. The slurry material was filtered in a Buchner funnel through filter paper and washed with 200 ml of the equilibration buffer. The resulting DEAE-cellulose cake was suspended in two volumes of equilibration buffer and poured into a glass column. The protein was eluted with 0.3 M $(NH_4)_2SO_4$ in buffer C, and the peak fraction was diluted with buffer C to 0.03 M $(NH_4)_2SO_4$. The diluted solution was chromatographed using a DEAE-Sephadex A-25 column (2.7×18 cm), as described earlier. Fractions which contained RNA polymerase I were diluted to 0.03 M $(NH_4)_2SO_4$ with buffer C and applied to a phospho-cellulose column (2.2×14 cm) equilibrated with 0 03 M $(NH_4)_2SO_4$ in buffer C. The column was eluted with a linear gradient of 180 ml from 0 03 M to 0 6 M $(NH_4)_2SO_4$ in buffer C, and 3.4 ml of fractions were collected. A half volume of glycerol was added to the enzyme fractions to bring the final glycerol concentration to about 50% and stored at −80°.

RNA polymerase assay. The reaction mixture contained 50 mM Tris-HCl, (pH 7.9) ; 1.6 mM $MnCl_2$; 8 mM KCl ; 2 mM 2-mercaptoethanol ; 0.4 mM each of ATP, GTP, and CTP ; 0.02 mM unlabeled UTP ; 1 μCi of [^{3}H] UTP (10–20 Ci/m mole) and 2.5 μg calf thymus DNA in a final volume of 0.2 ml. The enzyme fraction and $(NH_4)_2SO_4$ were added to the mixture as indicated in the legends of Figures and Tables. After incubation for 10 min at 37°, the reaction was stopped by cooling to 0°C and adding 2 ml of 5% trichloroacetic acid containing 0.04 M sodium pyrophosphate. After 1 h the acid-precipitable fraction was collected on a Whatman GF/C filter disc by suction. The filter was washed 4 times with 2 ml of cold trichloroacetic acid solution, dried under an infrared lamp, and counted in a scintillation vial with 10 ml of toluene containing PPO and POPOP. The counting efficiency was about 30%. A zero-time control value was subtracted from each count. One enzyme unit was defined as the incorporation of 1 n mole of UTP into the acid precipitable fraction in 10 min under standard conditions.

Chemical analysis. Protein was determined by Lowry's method after precipitation with cold 5% trichloroacetic acid (12). DNA was extracted from mouse liver and SR-C3H cells by the procedure of Kirby and Cook (11), and determined spectrophotometrically. The concentration of $(NH_4)_2SO_4$ was monitored by measuring the conductivity.

RESULTS

The results of the purification of RNA polymerases are shown in Table 1. RNA polymerases were eluted at 0.13 M and 0.24 M of $(NH_4)_2SO_4$ from DEAE-

TABLE 1. PREPARATION OF RNA POLYMERASES FROM SR-C3H CELL NUCLEI

	Total protein (mg)	Specific activity (units/mg)	Total activity (units)
Homngenate[a]	33	0.042	1.39
RNA polymerase I[b]	N.D.[c]	3.9[d]	1.84
RNA polymerase II[b]	N.D.[c]	2.7[d]	1.12

a Assayed at 0.05 M $(NH_4)_2SO_4$.
b Assayed at 0.06 M $(NH_4)_2SO_4$.
c Not determined.
d Specific activity was determined in the peak fractions.

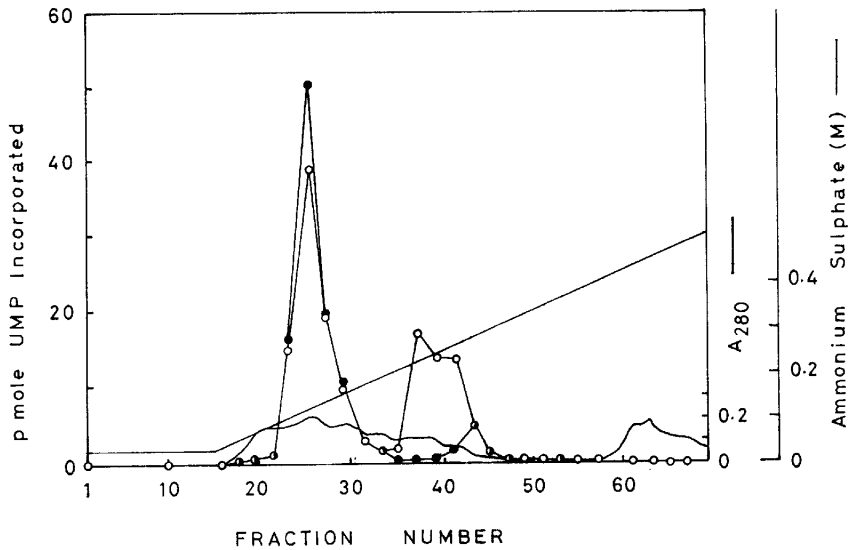

Fig. 1. DEAE-Sephadex chromatography of RNA polymerase isolated from SR-C3H cell nuclei. The supernatant fraction from the extract of 1.1 g nuclei was eluted from DEAE-Sephadex column (1.5×7.5 cm) with a gradient of $(NH_4)_2SO_4$. A 1.2 ml of elute was collected. ○——○, RNA polymerase activity in the abscence of α-amanitin; ●——●, RNA polymerase activity in the presence of 1 μg α-amanitin/ml.

Sephadex column. The first peak was not inhibited by 1 μg/ml of α-amanitin and the second peak was completely inhibited by 1 μg/ml of α-amanitin (Fig. 1 A). These peaks were RNA polymerases I and II, respectively. Polymerase activities obtained by DEAE-Sephadex chromatography were dependent on added DNA and inhibited by RNase. The reaction was enhanced by 50% with the addition of 10 mM 2-mercaptoethanol or 0.5 mM dithiothreitol (Table 2). After the reaction proceeded linearly for 30 min, the reaction velocity decreased gradually. The activity increased linearly with addition of enzyme.

TABLE 2. EFFECTS OF VARIOUS AGENTS ON RNA POLYMERASE FROM SR-C3H CELL NUCLEI

Conditions	RNA polymerase I (%)	RNA polymerase II (%)
Experiment 1		
plus cycloheximide	108	N. D.
plus actinomycin D (25 μg/ml)	5	1
plus α-amanitin (1 μg/ml)	94	17
plus DNase I (150 μg/ml)	5	28
plus RNase A (150 μg/ml)	0.3	3.7
minus DNA	0.4	1.5
Experiment 2		
plus mercaptoethanol (10 mM)	149	N. D.
plus dithiothreitol (0.5 mM)	158	N·D.

RNA polymerase activity was assayed under standard conditions, except for the above agents. RNA polymerase I or II (50 μl) was added to the assay, and $(NH_4)_2SO_4$ was adjusted to 0.05 M for RNA polymerase I and to 0.1 M for RNA polymerase II. 100% of the activity for experiment 1 represents the activity of standard reaction condition and 100% of activity for experiment 2 represents standard reaction conditions without mercaptoethanol or dithiothreitol.

Divalent cation requirement. Divalent cations were required for the reaction (Fig. 2). The optimal concentration of Mn^{++} ions for RNA polymerases I and II

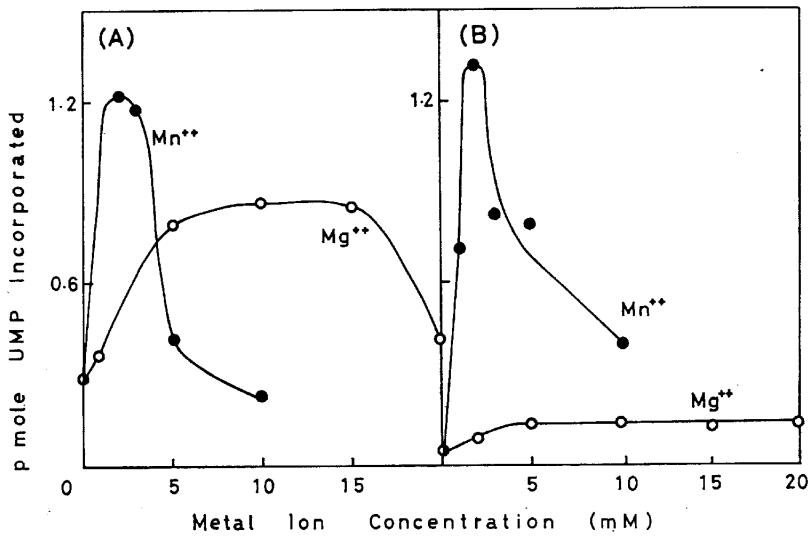

Fig. 2. Effect of divalent metal ion concentration on SR-C3H nuclear RNA polymerase. (A), Activity of RNA polymerase I. (B), Activity of RNA polymerase II. RNA polymerases I and II purified by DEAE-Sephadex chromatography were dialyzed against 0.03 M $(NH_4)_2SO_4$ in buffer C (see text), and 50 μl of the enzymes were assayed at the indicated concentrations of divalent cations. ●——●, manganese ion; ○——○, magnesium ion.

was about 2 mM. The optimal concentration of Mg^{++} was 5 to 15 mM. The ratio of activity assayed at optimal Mn^{++} ions to activity assayed at optimal Mg^{++} ions was 1.5 for RNA polymerase I and 9 for RNA polymerase II.

Effects of ionic strength. The reaction by the polymerases was markedly influenced by ionic strength (Fig. 3). RNA polymerase I had optimal activity at concentrations about 0.05 M $(NH_4)_2SO_4$ and RNA polymerase II had optimal activity at 0.1 M $(NH_4)_2SO_4$. At ionic strengths above 0.2 M $(NH_4)_2SO_4$ the activities of both polymerases were strongly inhibited.

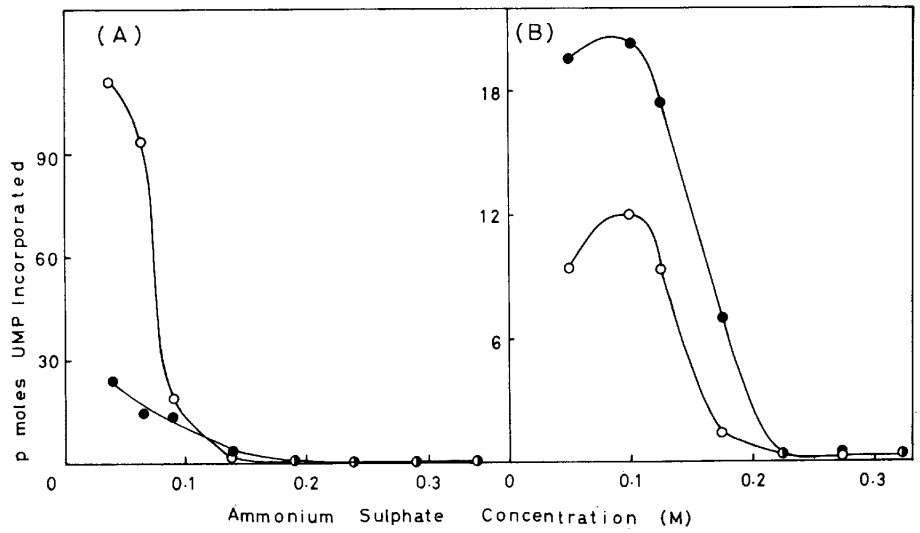

Fig. 3. Effect of ionic strength on RNA polymerase activity. (A), Activity of RNA polymerase I. (B), Activity of RNA polymerase II. RNA polymerases were dialyzed against 0.03 M $(NH_4)_2SO_4$ in buffer C (see text) and assayed at the indicated concentrations of $(NH_4)_2SO_4$. Calf thymus DNA was dissolved in 10 mM Tris-HCl, pH 7.9, 10 mM NaCl and 1 mM EDTA. Denaturation of DNA was performed by heating in a boiling water bath for 10 min and cooled rapidly. ○——○, assayed with native DNA; ●——●, assayed with denatured DNA.

Template requirement. RNA polymerase I transcribed native DNA four times more effectively than denatured DNA at 0.05 M $(NH_4)_2SO_4$. This ratio was reduced gradually with lower concentrations of $(NH_4)_2SO_4$ (Fig. 3). RNA polymerase II transcribed denatured DNA more effectively than native DNA.

The stability of the initiation complex formed with native or denatured DNA was tested by assaying the activity at various $(NH_4)_2SO_4$ concentrations after the formation of initiation complex (Fig. 4). The initiation complex between the template and the polymerases was formed by incubating the reaction mixture without UTP at 37°C for 10 min. After chilling the mixture, [^{3}H]

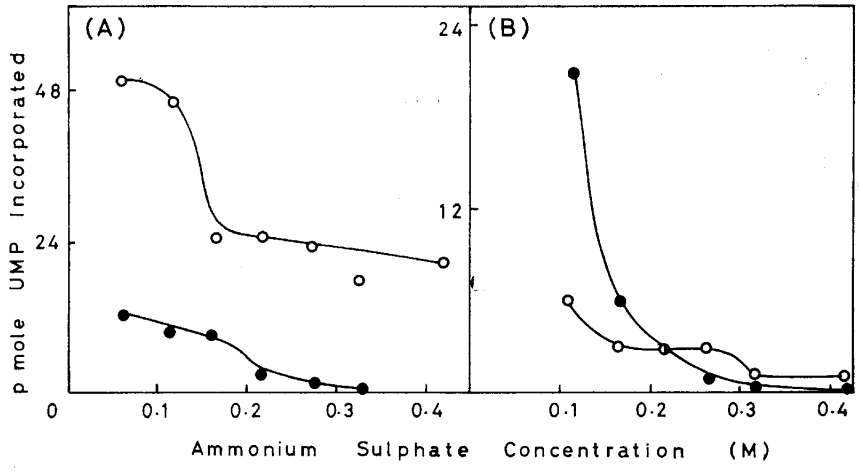

Fig. 4. Effect of salt concentration on the elongation reaction of RNA polymerase. (A), RNA polymerase I. (B), RNA polymerase II. Initiation complexes of RNA polymerase I or II with native or denatured DNA were formed in the RNA polymerase assay mixture without UTP and [^{3}H] UTP. $(NH_4)_2SO_4$ was added to the mixture to 0.02 M for RNA polymerae I and 0.12 M for RNA polymerase II. After incubation for 10 min at 37°, 1 μCi [^{3}H] UTP (15 Ci/m mole) and indicated amounts of $(NH_4)_2SO_4$ were added. These mixture were further incubated at 37° for 10 min. ○——○, chain elongation on native DNA; ●——●, chain elongation on denatured DNA.

UTP and various amounts of $(NH_4)_2SO_4$ were added to the reaction mixture and incubated further for the elongation reaction. The initiation complex with native DNA for both polymerases proceeded to the elongation reaction relatively insensitive to higher concentrations upto 0.2 M of $(NH_4)_2SO_4$ while the initiation process was mostly inhibited at 0.2 M as described earlier. Fig. 5 shows the elongation reaction assayed at 0.2 M $(NH_4)_2SO_4$ after formation of the initiation complex at low ionic strength. The elongation reaction of RNA polymerase I assayed with denatured DNA ended after 1 to 5 min whereas elongation with native DNA continued for 20 min. The elongation reaction of RNA polymerase II with denatured DNA was slowed down more rapidly than elongation with native DNA. These results demonstrate that a template of double stranded structure may be necessary for stable transcription although denatured DNA is a better template than native DNA for the initiation reaction of RNA polymerase II.

The template specificity of DNA from other sources was investigated by titration of the polymerases with various amounts and types of DNA (Fig. 6). Calf thymus DNA saturated DNA polymerase I at a lower concentration than mouse liver DNA or SR-C3H DNA. At optimal concentrations of these DNA, RNA polymerase I transcribed calf DNA more effectively than other DNAs, while RNA polymerase II transcribed mouse liver DNA or SR-C3H DNA more effectively than calf thymus DNA.

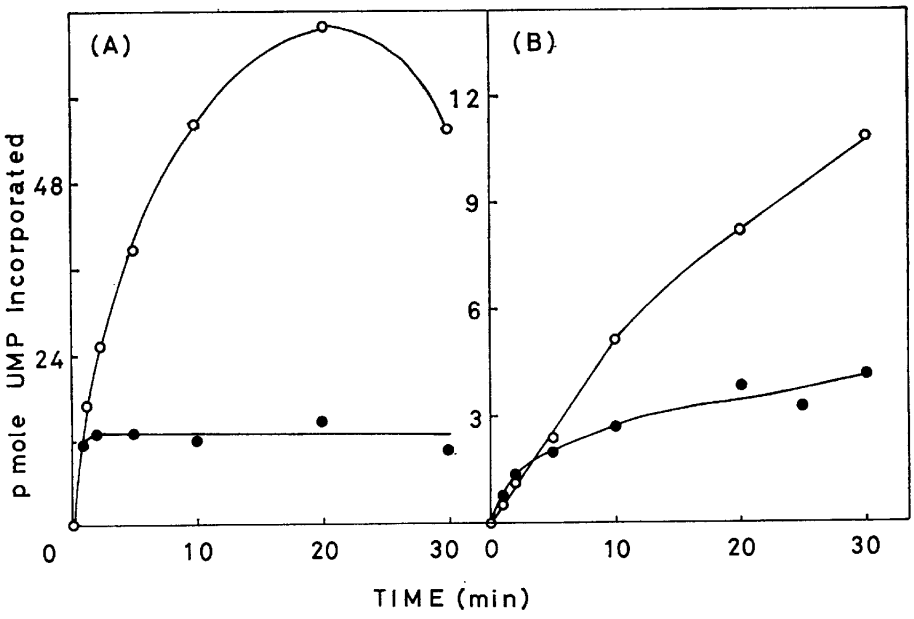

Fig. 5. Time course of RNA chain elongation by RNA polymerase. (A), RNA polymerase I. (B), RNA polymerase II. After initiation complex formation with native or denatured DNA as in Fig. 4, 1 μCi [^{3}H] UTP (15 Ci/m mole) was added and the $(NH_4)_2SO_4$ concentration was adjusted to 0.22 M. These mixtures were further incubated at 37° for the indicated time. ○——○, chain elongation on native DNA, ●——●, chain elongation on denatured DNA.

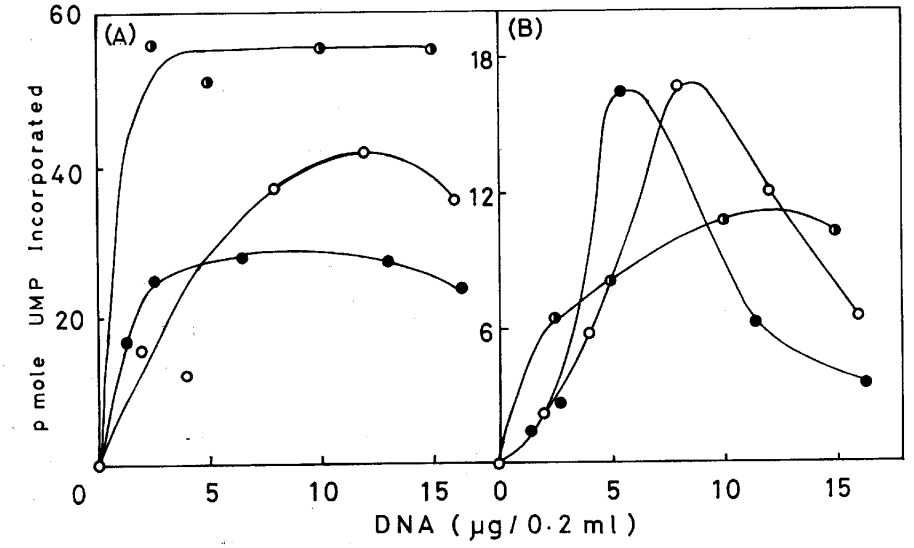

Fig. 6. Transcription of DNA from different sources. (A), activity of RNA polymerase I, (B), activity of RNA polymerase II. DNAs were dialyzed against 10 mM Tris-HCl, pH 7.9; 10 mM NaCl; and 0.1 mM EDTA. The indicated amounts of DNA were added to the standard reaction mixture. The concentration of $(NH_4)_2SO_4$ in the assay was 0.07 M for RNA polymerase I and 0.15 M for RNA polymerase II. ◑——◑, calf thymus DNA; ○——○, SR-C3H DNA; ●——●, mouse liver DNA.

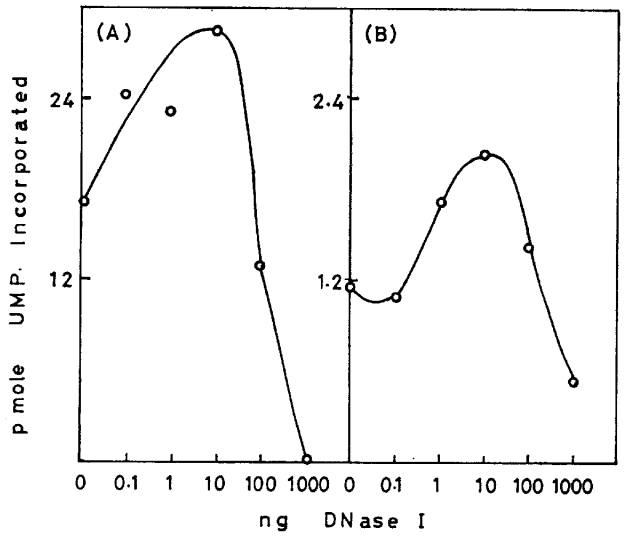

Fig. 7. Effect of partial digestion of DNA on transcription. (A), activity of RNA polymerase I. (B), activity of RNA polymerase II. DNA (2.5 μg) was digested in 0.1 ml of buffer containing 0.05 M Tris-HCl, pH 7.9; 0.01 M $MgCl_2$; 0.2 mM dithiothreitol; 2.5% glycerol; 0.05 M $(NH_4)_2SO_4$ and the indicated amounts of DNase I. After incubation for 20 min at 37°, the following components were added: 50 μl of RNA polymerase I or II; 50 μl of a mixture containing 0.1 M Tris-HCl, pH 7.9; 3.2 mM $MnCl_2$; 0.8 mM each of ATP, GTP and CTP; and 1 μ Ci [^{3}H] UTP (15 Ci/m mole). These mixtures were incubated for 20 min at 37°.

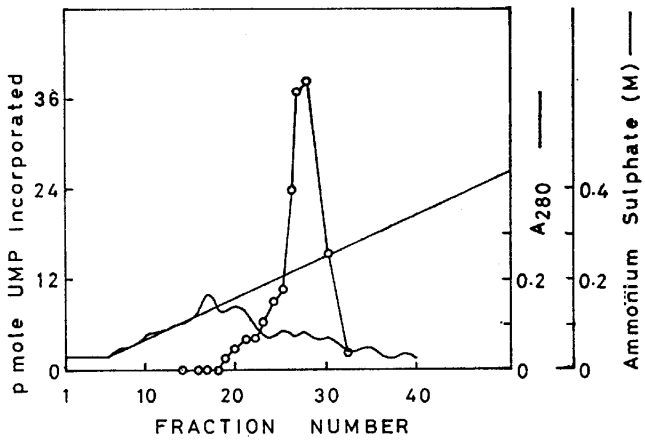

Fig. 8. Phospho-cellulose chromatography of RNA polymerase I isolated from SR-C3H cells. RNA polymerase was extracted from 52 g of SR-C3H cells. The active fraction eluted from DEAE-cellulose column was rechromatographed on DEAE-Sephadex. The fraction containing RNA polymerase I was chromatographed on phospho-cellulose column (2.2×14 cm) and 3.4 ml of elute were collected.

To test if single strand nicking of the template was preferable for the polymerase reaction, DNA previously digested with DNase I was used. Calf thymus DNA previously digested with various concentrations of pancreatic DNase I (19) were transcribed by RNA polymerases. Partial digestion of DNA by 10^{-2} μg of DNase I increased UTP incorporation into RNA synthesized by RNA polymerases I and II (Fig. 7). This result suggests that nicks in the DNA produced by low concentrations of DNase I (24) had the same effect on RNA polymerases I and II activities, and the difference in saturation of various DNA with RNA polymerases I and II (Fig. 6) may not be due to the nicking of DNA.

The main component of RNA polymerase I after phospho-cellulose chromatography was Ib (according to the nomenclature Chesterton and Butterworth (5)) (Fig. 8).

DISCUSSION

RNA polymerase from SR-C3H cells was resolved into two species that corresponded to RNA polymerase I, and II on DEAE-Sephadex chromatography. The activity of RNA polymerase I was relatively higher than that of RNA polymerae II. In SR-C3H cells RNA polymerae III was a smaller component, eluted as a shoulder after RNA polymerase II and was insensitive to 1μg/ml of α-amanitin. The properties of RNA III were not analyzed in this study. RNA polymerase I from rat liver nuclei contained mostly Ia form (4). Rat liver Ib enzyme was extracted with low salt concentrations at 37°C (5). RNA polymerae I from calf thymus did not show heterogenicity in phospho-cellulose chromatography and was demonstrated to be Ia form (or AI form) (9). RNA polymerase from *Xenopus laevis* was separated into two species by CM-Sephadex chromatography (16), but the relationship between *Xenopus* enzymes and SR-C3H enzymes is not clear.

The RNA polymerases I (Ib) and II from SR-C3H cells had characteristics similar to enzymes from other sources (1, 2, 13, 14). RNA polymerase I actively transcribed native DNA, and polymerase II actively transcribed denatured DNA. At higher ionic strength the initiation complexes of both enzymes with denatured DNA were unstable. These results suggest that the higher affinity of RNA polymerase II for denatured DNA was non-specific or non-specific termination of the reaction occurred. RNA polymerase II may require an initiation factor on native template (3, 10).

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