Journ. Fac. Sci., Hokkaido Univ. Ser. V (Botany), 12 (2): 111-122, 1980

A rapid and facile procedure for the purification of DNA-dependent RNA polymerases I and II of the cellular slime mold *Dictyostelium discoideum*

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A method was established for the purification of the DNA-dependent RNA polymerases I and II (ribonucleoside 5′-triphosphate: RNA nucleotidyltransferase, EC 2.7.7.6) of the cellular slime mold *Dictyostelium discoideum*. The enzymes were solubilized by sonication at a high ionic-strength from isolated nuclei and purified by batchwise treatment with DEAE-cellulose, ammonium sulfate precipitation, DEAE-Sephadex A-25 and DNA-cellulose column chromatography. The procedure was easily scaled up or down and afforded within 48 hr RNA polymerases I and II which were purified about 5,000 and 1,800 fold with yields of approximately 60% and 30%, respectively. The optimal concentrations of salts and divalent cations for the enzymes were similar to those of the polymerases in higher plants and mammalian cells.

Eukaryotic cells contain three structurally and functionally different classes of DNA-dependent RNA polymerases, designated as I, II and III (ROEDER, 1976). It is commonly thought that RNA polymerase I is responsible for the synthesis of the precursor to large ribosomal RNA's (BLATTI *et al.*, 1970; REEDER & ROEDER, 1972), RNA polymerase II synthesizes heterogeneous nuclear RNA (BLATTI *et al.*, 1970; EGYHAZI *et al.*, 1972), and RNA polymerase III is involved in the synthesis of 5 S rRNA, tRNA and some other low-molecular weight RNA's (WEINMANN & ROEDER, 1974; SKLAR & ROEDER, 1977). These multiple forms of RNA polymerase may have an important role in the selective transcription of the eukaryotic genome.

Purified RNA polymerases are required to study the mechanism of transcription *in vitro*. In *D. discoideum*, two types of RNA polymerases (I and II) have so far been reported (PONG & LOOMIS, 1973; YAGURA *et al.*, 1976, 1977). In this paper, we describe a method for the facile preparation of RNA polymerases I and II of this organism. The procedure involves batchwise treatment with DEAE-cellulose, ammonium sulfate precipitation,

Abbreviations: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid: SDS, sodium dodecyle sulfate.

DEAE-Sephadex and DNA-cellulose column chromatography. The characteristic of this method is that RNA polymerases I and II are relatively highly purified with the specific activity and yield, and the size of the preparation can easily be scaled up. We also report some properties of *D. discoideum* RNA polymerases I and II purified by this method and the evidence for the existence of RNA polymerase III.

Materials and Methods

Chemicals

[5-^{3}H] UTP (13.6 Ci/mmol) was purchased from the Radiochemical Centre, Amersham. ATP, GTP, CTP, UTP, α-amanitin, and rifampicin were from Boehringer Mannheim Biochemicals. Bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), and calf thymus DNA (type 1) were obtained from Sigma Chemical Co. DEAE-cellulose (DE 23) was from Whatman Ltd. and DEAE-Sephadex A-25 was from Pharmacia Fine Chemicals. Cellulose powder (Cellex 410 of Bio-Rad Lab.) was a gift from Dr. A. MATSUKAGE, Aichi Cancer Center Research Institute, and Esherichia coli RNA polymerase core enzyme was kindly provided by Dr. A. ISHIHAMA, Institute for Virus Research, Kyoto University.

DNA-cellulose was made from washed cellulose and native calf thymus DNA by the method of LITMAN (1968) with some modifications. The DNA-cellulose so prepared contained 6.05 mg of DNA per g of DNA-cellulose (dry weight).

Organisms and isolation of nuclei

D. discoideum strain NC 4 was grown in association with *E. coli* at 23°C on nutrient agar plates. The amoeba cells were harvested at the stage of interphase to aggregation and washed repeatedly with 14 mM phosphate buffer by centrifugation to remove bacterial cells. Nuclei were isolated according to the method described previously (YAGURA *et al.*, 1976; 1977) and then stocked at −80°C until use.

Assay for RNA polymerase activity

The standard assay mixture contained, in a total volume of 0.1 m*l*, 0.05 M Tris-HCl, pH 7.9, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5 mM each of ATP, GTP and CTP, 0.01 mM UTP, 1 μCi of [^{3}H] UTP, 1.5 mM $MnCl_2$, 5 mM $MgCl_2$, 10 μg of native calf thymus DNA and 100 μg bovine serum albumin.

The reaction was started by the addition of 50 μl of the enzyme, continued for 40 min at 23°C, and stopped by pipetting the reaction mixture

onto DEAE-cellulose papers (Whatman DE 81). They were washed six times with 5% (w/v) Na_2HPO_4 and twice each with water, ethanol and diethyl ether, according to the method of LINDELL *et al.* (1970), and counted with a Beckman liquid scintillation spectrometer. One unit of the enzyme activity was defined as 1 nmol of UMP incorporated under the above conditions.

Protein was determined after precipitation with 10% trichloroacetic acid by the method of LOWRY *et al.* (1951) with bovine serum albumin as a standard. DNA was measured by the method of BURTON (1956) with calf thymus DNA as a standard.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was carried out in slabs using the discontinuous Tris/glycine buffer system of LAEMMLI (1970). The molecular weight of the enzyme subunits was estimated from comparisons with those of the marker proteins run in parallel on separate gels.

Enzyme purification

Enzyme solubilization——All procedures were carried out at 0~4°C. About 5 g of frozen nuclei were lyzed and homogenized in 20 m*l* of buffer A (0.05 M Tris-HCl, pH 7.9, 1 mM EDTA, 10% glycerol, 20 mM 2-mercaptoethanol and 1 mM PMSF). The homogenate was adjusted to an ammonium sulfate concentration of 0.3 M by the addition of 4 M ammonium sulfate solution and sonicated 3 times for 15 sec with time intervals of more than 1 min. The sonicate was diluted with two volumes of buffer A and centrifuged for 30 min at 15,000×*g*. The resulting supernatant was diluted to 0.05 M ammonium sulfate with an equal volume of buffer A. The pale brownish extract (Fraction 1) was directly treated with DEAE-cellulose, as described below.

DEAE-cellulose treatment——To the above sonicated extract (Fraction 1) was added 4 g (dry weight) of DEAE-cellulose previously equilibrated in buffer A containing 0.05 M ammonium sulfate. The mixture was gently stirred for 40 min and centrifuged for 2 min at 8,000×*g*. After decanting the supernatant, the cellulose cake was resuspended in 100 m*l* of the above buffered solution, stirred for 10 min, and centrifuged. This procedure was repeated further 3 times. The last cake was suspended in 23 m*l* of buffer A containing 0.4 M ammonium sulfate (the salt concentration of the suspension was strictly corrected to 0.4 M) and filtered through nylon mesh in a column tube after stirring for 40 min. The filtrate so obtained contained the RNA polymerase (Fraction 2).

DEAE-Sephadex chromatography——To the above filtrate (Fraction 2)

was added 1.5 volumes of saturated ammonium sulfate solution prepared with buffer B (0.05 M Tris-HCl, pH 7.9, 0.5 mM EDTA, 10% glycerol), and the mixture was left overnight in an ice bath. The resulting precipitate was collected by centrifugation for 1 hr at 70,000×*g*, suspended in 2.5 m*l* of buffer C (0.05 M Tris-HCl, pH 7.9, 0.5 mM EDTA, 25% glycerol) containing 0.05 M ammonium sulfate and 10 mM 2-mercaptoethanol and then dialyzed for 5 hr against 2 liters of the above buffered solution with 3 changes. The dialysate (Fraction 3) was applied to a DEAE-Sephadex A-25 column (1.5×10 cm) previously equilibrated with buffer C containing 0.05 M ammonium sulfate and 2 mM DTT. The column was washed with 3 bed volumes of the above buffered solution and RNA polymerases were eluted with 120 m*l* of a linear gradient from 0.05 M to 0.3 M ammonium sulfate in buffer C containing 2 mM DTT. Fractions having polymerase activity were collected and pooled for the next procedure.

DNA-cellulose chromatography——The fractions of RNA polymerase I (22 m*l*) and II (22 m*l*) were dialyzed against 2 liters of buffer D (0.02 M Tris-HCl, pH 7.9, 0.1 mM EDTA, 25% glycerol) containing 0.05 M KCl and 10 mM 2-mercaptoethanol with two changes. The dialysate was loaded on native DNA-cellulose columns (0.6×5 cm) previously equilibrated with buffer D containing 0.05 M KCl and 1 mM DTT. The columns were washed with 15 m*l* of the above buffered solution and the enzymes were eluted stepwise with 15 m*l* each of 0.3 M and 0.5 M KCl in buffer D containing 1 mM DTT. Fractions containing the enzyme activity were pooled and then used as the purified enzyme.

Results and Discussion

The procedure for the purification of RNA polymerases I and II is summarized in Table I. Almost all of the enzyme activity was solubilized at high-ionic strength by sonication of less than 1 min. A longer sonication time resulted in a loss of the enzyme activity (data not shown).

Figure 1 shows a typical elution pattern of the ammonium sulfate-precipitated enzymes (Fraction 3) on a DEAE-Sephadex column. Two enzyme activity peaks were observed at about 0.12 M and 0.18 M ammonium sulfate under the standard assay conditions. However, when the activity was assayed in the presence of α-amanitin (3 μg/m*l*), the activity peak at 0.18 M ammonium sulfate disappeared and two small peaks appeared at about 0.17 M and 0.22 M ammonium sulfate. The enzyme eluted at 0.17 M ammonium sulfate was probably RNA polymerase of *E. coli* used as food for culturing of the amoebae, because it was sensitive to rifampicin (30 μg/m*l*) and not

TABLE 1. Summary of purification of RNA polymerases I and II of *D. discoideum*[a]

Purification step		Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Sonication	I[b]	548.33	507.9	0.93	1	100
	II[c]		1491.4	2.72	1	100
Cemtrifugation (15,000×*g*, 30′)	I[b]	333.00	639.5	1.92	2	124
	II[c]		1049.3	3.15	1	70
DEAE-cellulose batch	I[b]	13.098	348.0	26.57	29	66
	II[c]		783.3	59.80	22	53
DEAE-Sephadex column	I[b]	1.907	287.6	150.80	162	57
	II[c]	2.328	843.7	362.41	133	57
DNA-cellulose column	I	0.056	305.0	5446.43	5856	60
	II	0.090	436.6	4818.42	1771	29

a) Enzyme activities and yields are given from a single representative experiment. About 5 g (wet weight) of nuclei isolated from cells at the interphase-aggregation stage was used as starting materials.
b) Activity resistant to 3 μg/m*l* α-amanitin in the presence of 30μg/m*l* rifampicin.
c) Activity sensitive to 3 μg/m*l* α-amanitin.

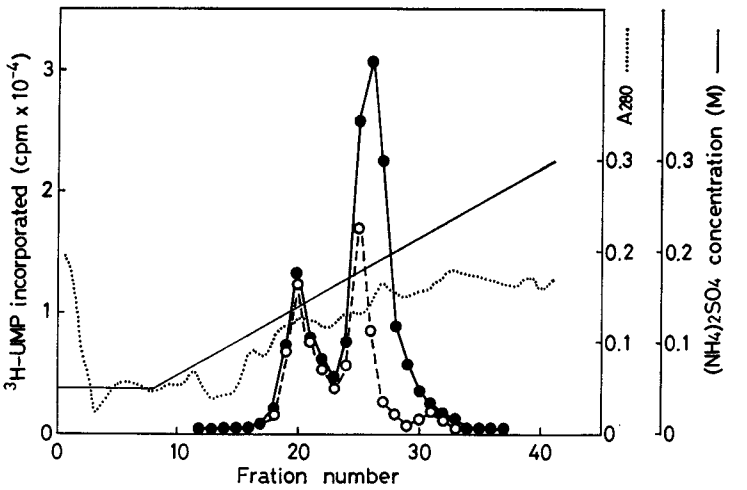

Fig. 1. DEAE-Sephadex A-25 column chromatography of *D. discoideum* RNA polymerases partially purified by DEAE-cellulose batchwise treatment and the subsequent ammonium sulfate precipitation procedure. The enzymes were prepared at the interphase-aggregation stage and loaded on a DEAE-Sephadex A-25 column (1.5×10 cm). After washing the column, the enzyme was eluted with a linear gradient of $(NH_4)_2SO_4$. Fractions of 4 m*l* each were collected at the flow rate of 15 m*l*/hr and assayed for RNA polymerase activity in the presence (○——○) or absence (●——●) of 3 μg/m*l* α-amanitin. All other conditions for the purification, chromatography, and assay of enzymes are described in Materials and Methods.

found in the cell sample completely free of *E. coli* cells. The rifampicin-sensitive RNA polymerase could be completely separated from *D. discoideum* polymerases by affinity chromatography on a DNA-cellulose column (Fig. 2). The activity peak at 0.22 M was probably RNA polymerase III, judging from the elution profile of the enzyme from the DEAE-Sephadex column and the mode of its sensitivity to α-amanitin, as described below. According to the same criteria, the enzymes eluted at 0.12 M and 0.18 M ammonium sulfate were RNA polymerases I and II, respectively, as already reported (YAGURA *et al.*, 1977).

RNA polymerases I and II were purified by affinity chromatography on

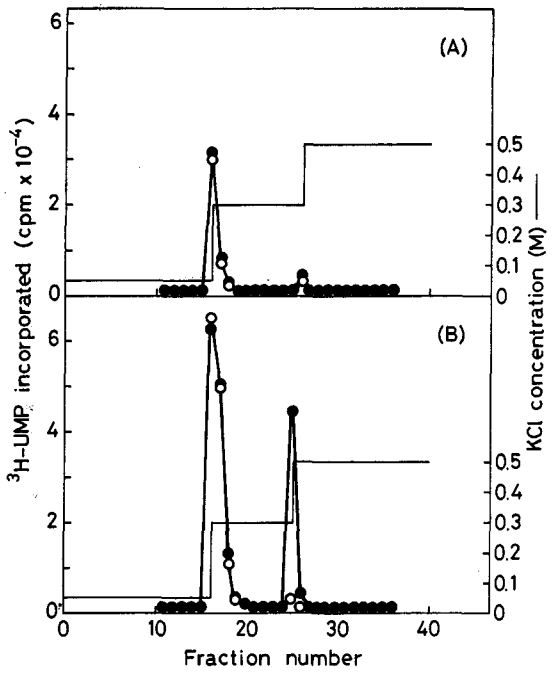

Fig. 2. DNA-cellulose column chromatography of *D. discoideum* RNA polymerases I and II after purification on DEAE-Sephadex columns. Fractions containing RNA polymerase I and II activities obtained by DEAE-Sephadex chromatography were separately pooled, dialyzed against buffer D containing 0.05 M KCl and 10 mM 2-mercaptoethanol, and applied to native calf thymus DNA-cellulose columns (0.6×5 cm). After washing the columns, the enzyme was eluted stepwise with 15 m*l* each of 0.3 M and 0.5 M KCl. Fractions of 1.5 m*l* each collected and assayed for RNA polymeyase activity in the presence (○——○) or absence (●——●) of 30 μg/m*l* rirampicin. All other conditions for the purification, chromatography, and assay of enzymes are described in Materials and Methods. A, RNA polymerase I; B, RNA polymerase II.

DNA-cellulose columns. Figure 2 shows the typical chromatographic profiles of both the enzymes, in which they were completely eluted with 0.3 M KCl. The enzyme eluted with 0.5 M KCl may be contaminating *E. coli* RNA polymerase, because it was resistant to α-amanitin but sensitive to rifampicin. Thus, *D. discoideum* RNA polymerases I and II became completely free from bacterial RNA polymerase with this affinity chromatography.

Little DNA was contained in the final enzyme fraction, as far as it was checked colorimetrically. The specific activities of RNA polymerases I and II which were purified about 5,000 and 1,800 fold were about 5,400 and 4,800 units/mg of enzyme protein, respectively. The values are comparable with those of purified RNA polymerases of other eukaryotes (HARGER *et al.*, 1977; SMITH & BRAUN, 1978; SPINDLER *et al.*, 1978).

Figures 3 and 4 show the effect of divalent cations and salts on the polymerase activity. The optimal concentration of $MnCl_2$ was 1.5 mM for RNA polymerases I and II, and that of $MgCl_2$ was 2.5 mM for polymerase

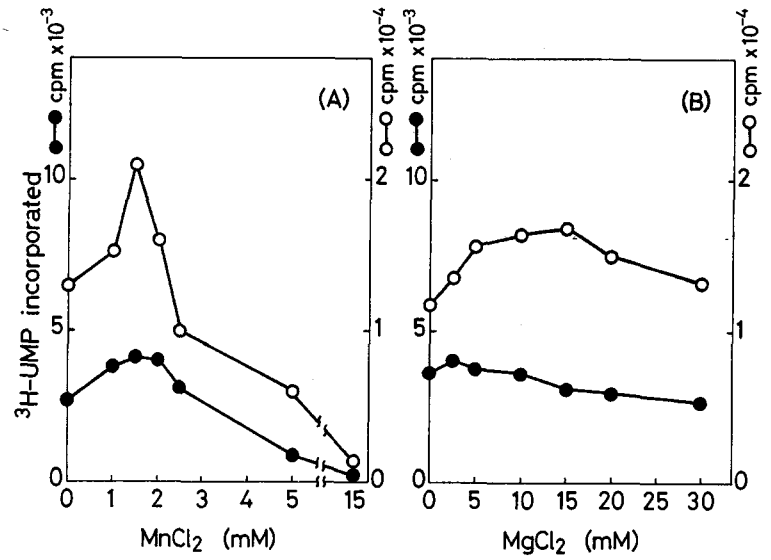

Fig. 3. Effect of the divalent metal ions, Mn^{2+} and Mg^{2+} on the activities of *D. discoideum* RNA polymerases I and II. The enzymes were obtained from cells at the interphase-aggregation stage. The assay conditions were the same as for the standard assay except for the concentrations of the metal ions indicated. The assay mixture contained 25 mM and 50 mM $(NH_4)_2SO_4$ for polymerases I and II, respectively. A, Effect of Mn^{2+}. MgCl concentration was 5 mM for polymerase I and 15 mM for polymerase II. B, Effect of Mg^{2+}. $MnCl_2$ concentration was 2 mM for both the enzymes. (●——●) RNA polymerase I; (○——○) RNA polymerase II.

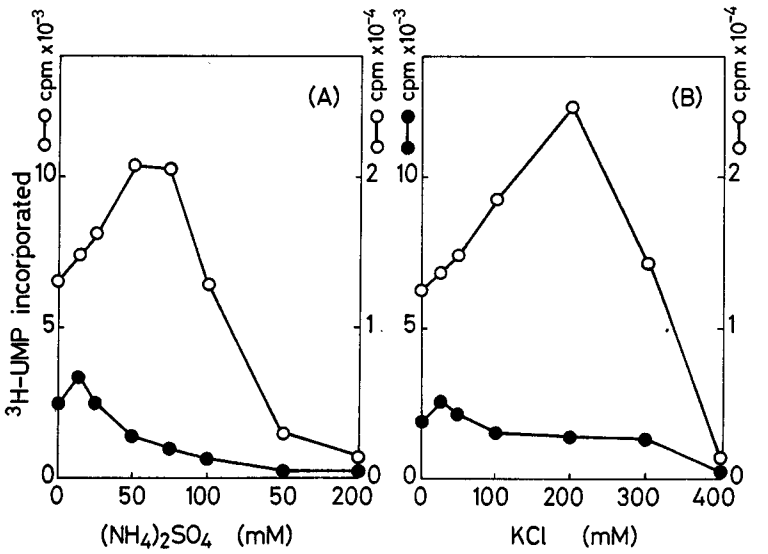

Fig. 4. Effect of $(NH_4)_2SO_4$ and KCl on the activities of *D. discoideum* RNA polymerases I and II. The enzymes were obtained from cells at the interphase-aggregation stage. The reaction mixture contained 5 mM $MgCl_2$ for the assay of polymerase I and 15 mM for that of polymerase II and 2 mM $MnCl_2$ for that of both enzymes. Other conditions were the same as in the experiments shown in Fig. 3. A, Effect of $(NH_4)_2SO_4$. B, Effect of KCl. (●——●) RNA polymerase I; (○——○) RNA polymerase II.

I and 15 mM for polymerase II (Fig. 3). With the effect of ammonium sulfate, the enzyme activity was maximum at 15 mM ammonium sulfate for polymerase I and 50 mM for polymerase II (Fig. 4). The concentration of KCl was optimum at 25 mM for the polymerase I activity and at 200 mM for the polymerase II activity. The above results were similar to those reported previously with partially purified enzymes (YAGURA *et al.*, 1976, 1977).

Figure 5 shows the α-amanitin sensitivity of polymerases I, II and III obtained after DEAE-Sephadex chromatography. The polymerase I activity was resistant to a high concentration (200 μg/m*l*) of α-amanitin, whereas the polymerase II activity was completely inhibited by a low concentration (0.3 μg/m*l*) of the drug. The concentration of the drug necessary to bring about 50% inhibition of the enzyme activity was about 0.005 μg/m*l* for polymerase II and about 30 μg/m*l* for polymerase III. The mode of α-amanitin sensitivity of *D. discoideum* RNA polymerases resembles that of mammalisn or higher plant RNA polymerases rather than that of yeast RNA polymerases.

The subunit structure of the enzymes was examined by polyacrylamide

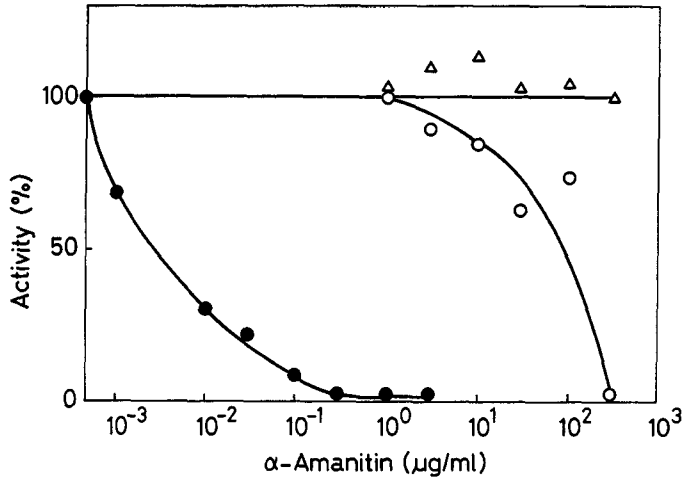

Fig. 5. Effect of α-amanitin on the activities of *D. discoideum* RNA polymerases I, II and III. Fractions containing the activities of RNA polymerases I, II and III were collected at the step of DEAE-Sephadex chromatography. The α-amanitin sensitivity of the enzymes was examined under the optimal conditions for each enzyme. For the measurements of the activities of polymerases II and III, rifampicin (30 μg/ml) was further added to the reaction mixture to suppress the activity of slightly contaminating *E. coli* RNA polymerase. One hundred percent of the activities of polymerases I, II and III represent 33.0, 334.6 and 4.5 pmoles, respectively, of UMP incorporated. (△——△) RNA polymerase I; (●——●) RNA polymerase II; (○——○) RNA polymerase III.

gel electrophoresis in the presence of SDS. As Fig. 6 shows, 7 and 6 protein bands were observed with RNA polymerases I and II obtained after DNA-cellulose chromatography, respectively, when 9% acrylamide gels were used. The subunits having molecular weights of less than 22,000 could be resolved by using 12% gels into protein bands of molecular weights of 21,000, 16,500, 15,000 and 14,000 for RNA polymerase I, and those of molecular weights of 21,000, 16,500 and 15,000 for RNA polymerase II (data not shown). Thus, 10 and 8 subunits were finally identified for polymerase I and II, respectively. The subunit structure observed here was not essentially inconsistent with that reported by PONG and LOOMIS (1973), although there were some minor differences between their estimation and ours. Similar subunit structures were observed with RNA polymerases I and II of other eukaryotes (SKLAR *et al.*, 1975; GUILFOYLE, 1976; HAGER *et al.*, 1977; JENDRISAK & GUILFOYLE, 1978; SPINDLER *et al.*, 1978).

In this paper, we have described a method useful for the purification of RNA polymerases of *D. discoideum*. This method gave a good results

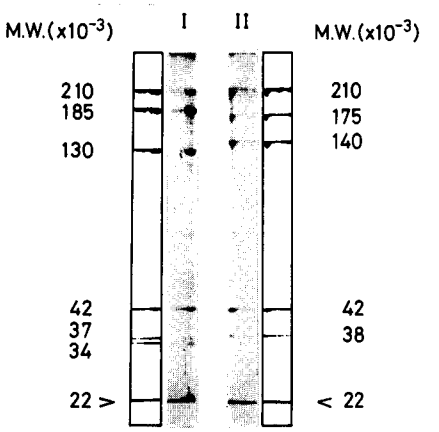

Fig. 6. SDS polyacrylamide gel electrophoresis of *D. discoideum* RNA polymerases I and II. The proteins of RNA polymerases I and II prepared from cells at the interphase-aggregation stage, as described in Materials and Methods was precipitated in the presence of polyadenylic acid (20 μg/m*l* at the final concentration) as a coprecipitator by the addition of 0.1 vol. of 100% TCA. The precipitate was collected, washed dried, and then dissolved in the sample buffer containing 3% SDS and 5% 2-mercaptoethanol. The protein solution was heated at 90°C for 5 min and then electrophoresed on 9% polyacrylamide slab gels in the presence of 0.1% SDS. Gels were stained with 0.05% coomassie brilliant blue in methanol/CH_3COOH/water (50 : 10 : 50, v/v) overnight and destained with 7% CH_3COOH. The marker proteins used were *E. coli* RNA polymerase β' (165,000) and β (155,000) subunits, bovine serum albumin (68,000), γ-globulin H-chain (58,000), ovalbumin (53,000), *E. coli* RNA polymerase α subunit (40,000) and γ-globulin L-chain (22,000).

for preparing RNA polymerases I and II of a considerably high specific activity, but was not applicable for the purification of polymerase III which was apt to leak out of nuclei during their isolation. Particularly, we must emphasize the advantage of this method in that purified RNA polymerases I and II can be prepared within 48 hr. The enzymes prepared by this method have been acturally employed to study their template specificity for synthetic polynucleotide polymers (TAKIYA *et al.*, in press).

We are grateful to Professor Ititarô HARADA for his encouragement in this study. We wish to thank to Professor Shoji SASAKI for facilitating use of the sonicator. Thanks are also due to Dr. Akio MATSUKAGE, Aichi Cancer Center Research Institute, for his gift of some chemical reagents and to Dr. Akira ISHIHAMA of Kyoto University for his gift of *E. coli* RNA polymerase. This work was supported in part by a grant (No. 334039) from the Ministry of Education of Japan.

References

BLATTI, S. P., INGLES, C. J., LINDELL, T. J., MORRIS, P. W., WEAVER, R. F., WEINBERG, F. and RUTTER, W. J. 1970. Structure and regulatory properties of eucaryotic RNA polymerase. Cold Spring Harbor Symp. Quant. Biol. **35**: 649-659.

BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**: 315-323.

EGYHAZI, E., D'MONTE, B. and EDSTROM, J.-E. 1972. Effect of α-amanitin on *in vitro* labeling of RNA from defined nuclear components in salivary gland cells from *Chironomus tentanis*. J. Cell Biol. **53**: 523-531.

GUILFOYLE, T. J. 1976. Purification and characterization of DNA-dependent RNA polymerases from califower nuclei. Plant Physiol. **58**: 453-458.

HAGER, G. L., HOLLAND, M. J. and RUTTER, W. J. 1977. Isolation of ribonucleic acid polymerases I, II and III from *Saccharomyces cerevisiae*. Biochemistry **16**: 1-8.

JENDRISAK, J. and GUILFOYLE, T. J. 1978. Eukaryotic RNA polymerases: comparative subunit structures, immunological properties, and α-amanitin sensitivities of the class II enzymes from higher plants. Biochemistry **17**: 1322-1327.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685.

LINDELL, T. J., WEINBERG, F., MORRIS, P. W., ROEDER, R. G. and RUTTER, R. J. 1970. Specific inhibition of nuclear RNA polymerase II by α-amanitin. Science **170**: 447-449.

LITMAN, R. M. 1968. A deoxyribonucleic acid polymerase from *Micrococcus luteus* (*Micrococcus lysodeikiticus*) isolated on deoxyribonucleic acid-cellulose. J. Biol. Chem. **243**: 6222-6233.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **248**: 3933-3939.

REEDER, R. H. and ROEDER, R. G. 1972. Ribosomal RNA synthesis in isolated nuclei. J. Mol. Biol. **67**: 433-441.

ROEDER, R. G. 1976. Eukaryotic nuclear RNA polymerases. In RNA polymerase (LOSICK, R. and CHAMBERLIN, M., Eds.), pp. 227-246, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

SKLAR, V. E. F., SCHWARTS, L. B. and ROEDER, R. G. 1975. Distinct molecular structures of nuclear class I, II and III DNA-dependent RNA polymerases. Proc. Natl. Acad. Sci. U. S. A. **72**: 348-352.

——— and ROEDER, R. G. 1977. Transcription of specific genes in isolated nuclei by exogenous RNA polymerase. Cell **10**: 405-414.

SMITH, S. S. and BRAUN, R. 1978. A new method for the purification of RNA polymerase II (or B) from the lower eukaryote *Physarum polycephalum*. Eur. J. Biochem. **82**: 309-320.

SPINDLER, S. R., DUESTER, G. L., D'ALESSIO, J. M. and PAUL, M. R. 1978. A rapid

and facile procedure for the preparation of RNA polymerase I from *Acanthamoeba castellanii*. J. Biol. Chem. **253**: 4669–4675.

WEINMANN, R. and ROEDER, R. G. 1974. Role of DNA-dependent RNA polymerase III in the transcription of the tRNA and 5S RNA genes. Proc. Natl. Acad. Sci. U. S. A. **71**: 1790–1794.

YAGURA, T., YANAGISAWA, M. and IWABUCHI, M. 1976. Evidence for two α-amanitin-resistant RNA polymerases in vegetative amoebae of *Dictyostelium discoideum*. Biochem. Biophys. Res. Commun. **68**: 183–189.

———, ——— and ———. 1977. Some properties of partially purified DNA-dependent RNA polymerases and changes of levels of their activities during development in *Dictyostelium discoideum*. J. Fac. Sci. Hokkaido Univ. V **10**: 219–230.