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Solubilization and Partial Purification of RNA Polymerase from Pea Chloroplasts†

K. K. Tewari* and A. Goel

ABSTRACT: DNA-dependent RNA polymerase of pea chloroplasts is tightly bound to a complex that contains chloroplast DNA, DNA polymerase, RNA polymerase, and many other proteins. This complex transcribes all the sequences of chloroplast DNA in vitro. The RNA polymerase has been solubilized and purified from this complex by fractionation in glycerol gradients and in DEAE-cellulose and Sepharose 6B columns. The purified enzyme was completely dependent on the exogenous DNA. The native molecular size of the enzyme was found to be more than 500 000 by native gel electrophoresis and glycerol gradients. Chloroplast RNA polymerase was obtained in 1500-fold purification, starting from the Triton-disrupted chloroplasts. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis has shown that chloroplast RNA polymerase may contain polypeptides of 180, 140, 110, 95, 65, 47, and 27 kdaltons. The enzyme has been found to be extremely sensitive to salt concentrations and had a temperature optimum of 30 °C. The purified enzyme is not inhibited by either α-amanitin or rifampicin.

The structural organization of the pea chloroplast (ct) genome has been extensively studied (Tewari, 1979). Pea ctDNA was found to contain one rRNA gene and 30–40 tRNA genes (Meeker & Tewari, 1980). The hybridization of pea ctDNA with ct polysomal RNA showed that RNA molecules equivalent to a complete single-stranded chain of ctDNA were transcribed in vivo (Oishi et al., 1981). The ribosomal RNA gene was localized in the restriction-endonuclease map of pea ctDNA (Chu et al., 1981; Palmer & Thompson, 1981). The gene for the large subunit of ribulose-bisphosphate carboxylase and two photodependent genes also were identified and mapped in pea ctDNA (Oishi & Tewari, 1983). So that one can understand the mechanism of transcription of the stable and messenger RNA genes of pea ctDNA, purified DNA-dependent RNA polymerase must be obtained from chloroplasts to develop an in vitro transcription system that faithfully mimics the in vivo transcription of ctDNA.

DNA-dependent RNA polymerase from chloroplasts of higher plants was found to be tightly bound with ctDNA and the thylakoid membranes (Tewari & Wildman, 1969). Early attempts to solubilize the enzyme from the membrane were unsuccessful despite hypotonic treatment, homogenization, freezing and thawing, salt extraction, or detergent treatment. Similar attempts with other higher plants were not successful. Hallick et al. (1976) purified a complex from *Euglena* that contained RNA polymerase bound to ctDNA. This complex, named a transcriptionally active chromosome, was found to carry out chain elongation and chain initiation. The RNA polymerase could not be dissociated from the bound ctDNA of this transcription complex. However, Bottomley et al. (1971) were successful in solubilizing and purifying the RNA polymerase from corn leaves. The critical step in obtaining the soluble RNA polymerase from corn was extraction of the membranes in the presence of 4 mM EDTA[1] at 37 °C. The purified ctRNA polymerase was found to contain a number of polypeptides at different stages of purification, but at least two polypeptides of 180 and 140 kdaltons always were found to be associated with the chloroplast RNA polymerase (Smith & Bogorad, 1974). Kidd & Bogorad (1979) compared 180-kdalton subunits from maize chloroplast and type II nuclear DNA-dependent RNA polymerase; but despite similar molecular mass, these 180-kdalton subunits were not the same as shown by one- and two-dimensional peptide mapping techniques. Data from these investigators also showed that the 160-, 43-, and 28-kdalton polypeptides from maize type II nuclear RNA polymerase, and 140-, 42-, and 27-kdalton polypeptides from maize chloroplast RNA polymerase, are unique. The purified RNA polymerase of maize was found to preferentially transcribe maize ctDNA sequences incorporated in cloned chimeric bacterial plasmids (Jolly & Bogorad, 1980). Preferential transcription was found to be dependent on the presence of a 27.5-kdalton polypeptide and the template being in the supercoiled form.

In this paper, we report on the solubilization and purification of RNA polymerase from pea chloroplasts. The enzyme was obtained in 1500-fold purification from Triton-disrupted

† From the Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717. *Received August 31, 1982.*

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; SSC, standard saline citrate; DEAE, diethylaminoethyl.

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chloroplast. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the enzyme showed it to be multimeric, containing at least six polypeptides of 180, 140, 110, 95, 65, 47, and 27 kdaltons.

Materials and Methods

Isolation of Chloroplasts. Pea leaves from 7–10 day old plants were razor chopped in 250-g lots with 500 mL of SM buffer containing 50 mM Tris-HCl, pH 8.0, 0.5 M sucrose, 5 mM $MgCl_2$, 10 mM mercaptoethanol, and 0.2 mM PMSF. The homogenate was filtered through four layers of cheesecloth and four layers of miracloth and centrifuged at 1000*g* for 10 min. The pellet was resuspended in SM buffer (about 250 mL/kg of pea leaves) and centrifuged again at 1000*g* for 10 min. The chloroplast fraction again was suspended in SM buffer (125 mL/kg of pea leaves), and 10 mL of the suspension was layered on top of discontinuous sucrose gradients consisting of 10 mL of 60%, 10 mL of 45%, and 5 mL of 25% sucrose (buffered with 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 10 mM mercaptoethanol, and 0.2 mM PMSF). After centrifugation for 2 h at 27 000 rpm in the SW27 rotor, the chloroplasts were collected from the 45% sucrose layers, diluted with an equal volume of 50 mM Tris-HCl, pH 8.0, and centrifuged at 1500*g* for 15 min. The pure chloroplast fraction also was obtained from the 1000*g* pellet by suspending it in SM buffer (200 mL/kg of pea leaves) and adding 50 mL of 12.5% Triton X-100. The nuclei were removed by centrifugation at 6000*g* for 15 min. The supernatant (TS) was found to be essentially free of nuclear contamination. We routinely isolated pure ctDNA from this fraction by substituting 5 mM EDTA for 5 mM $MgCl_2$ in the SM buffer.

RNA Polymerase Assay. The enzymatic activity was assayed in a 0.1-mL mixture containing 10 mM Tris-HCl, pH 8.5, 0.1 mM each of ATP, GTP, and CTP, and 1 μCi of [^{32}P]UTP (25 μM, 400 Ci/mmol). Specific activity of the enzyme was determined by substituting 0.25 μCi of [^{14}C]UTP (25 mCi/mmol) for [^{32}P]UTP. After incubation at 30 °C for 30 min, the reaction was stopped by adding 5.0 mL of cold 5% Cl_3CCOOH. The precipitated nucleic acids were filtered through glass-fiber filters. The filters were washed 3 times with 10 mL of 5% Cl_3CCOOH and twice with 10 mL of 80% ethanol. Radioactivity was determined in a scintillation counter.

Nucleic Acids. Nuclear and ctDNAs were isolated as described previously (Chu et al., 1981). Restriction endonucleases *Sal*I, *Sma*I, *Hin*dIII, and *Eco*RI were purchased from Bethesda Research Laboratories, Bethesda, MD. One to two micrograms of ctDNA was digested with specific endonucleases according to the optimum conditions for each enzyme. The DNA fragments produced by restriction endonucleases were analyzed for their molecular sizes by gel electrophoresis in 1% agarose. Electrophoresis was carried out for 16 h in a buffer containing 40 mM Tris-HCl, pH 7.8, 10 mM sodium acetate, 2 mM EDTA, and 0.5 mg/mL ethidium bromide. The DNA bands were visualized by illuminating with long-wave UV light, and DNA fragments were transferred to nitrocellulose filters according to Southern (1975). For hybridization with RNA, the filters were prehybridized with 0.08% each of Ficol, poly(vinylpyrrolidone), and BSA. Hybridizations were carried out in 40% formamide in 1× SSC at 37 °C. After hybridization, the nitrocellulose filters were washed in 1× SSC at room temperature and then treated with 10 μg of RNase A and 10 units of RNase T_1/mL for 1 h in 1× SSC at 37 °C. The filters were again extensively washed in 1× SSC at room temperature and then exposed to X-ray film.

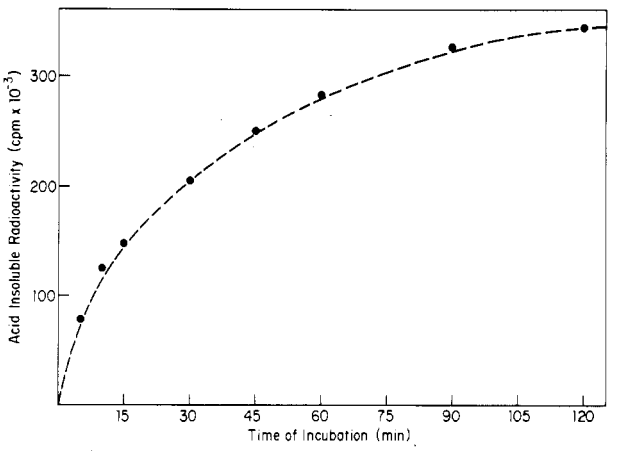

FIGURE 1: RNA polymerase activity of pea chloroplasts. Triton-disrupted chloroplasts from 1 kg of leaves were centrifuted at 65000*g* for 2 h. The final pellet as described in the text was suspended in 10 mL of TG buffer. The incubation was carried out in a final volume of 5 mL, which contained 1 mM each of ATP, GTP, and CTP, 500 μCi of [^{32}P]TUP, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.0, and 0.5 mL of the enzyme suspension. 0.1-mL aliquots at different time intervals were processed for the acid-insoluble radioactivity. After 5 min of incubation, 2.0 mL of the incubation mixture was removed for the extraction of RNA. Another aliquot of 1.0 mL was taken for RNA extraction after 60 min of incubation.

Results

RNA Polymerase Activity of Chloroplasts. The RNA polymerase activity of gradient-purified chloroplasts was not dependent upon exogenous DNA as observed before (Tewari & Wildman, 1969). When gradient-purified chloroplasts were disrupted with 2.5% Triton X-100 and centrifuged at 65000*g* for 2 h, the RNA polymerase activity was found to be associated with the pellet. About 70–85% of the total chloroplast polymerase activity could be recovered in this fraction. When the chloroplast fraction was purified by Triton treatment (see Materials and Methods), the RNA polymerase activity of the TS fraction also could be recovered in the 65000*g* pellet. The 65000*g* pellet was suspended in TM buffer (24 mL/kg of leaves) and the suspension was made to contain 2.5% Triton X-100 by adding 6 mL of 12.5% Triton. This suspension was again centrifuged at 65000*g* for 2 h. Eighty percent of the RNA polymerase activity of the first 65000*g* pellet was recovered in the pellet. The pellet was solubilized by suspension for 3 days in TG buffer containing 25% glycerol, 50 mM Tris-HCl, pH 8.5, 10 mM mercaptoethanol, and 0.2 mM PMSF. The solubilized fraction was still dependent upon endogenous DNA. In order to find out whether this fraction represented a true transcription complex of chloroplast, it was necessary to transcribe the RNA from the endogenous ctDNA by incubating this fraction under conditions necessary for RNA synthesis. The rate of RNA synthesis by this fraction is shown in Figure 1. Aliquots of the mixture were removed after 5 and 60 min, and the reaction was stopped by adding 0.5 mL of 10% $NaDodSO_4$. The solution was treated with 50 μg/mL Protease K and phenol extracted, and the RNA was precipitated from the aqueous phase by adding 2 volumes of ethanol and 100 μg of yeast RNA as a carrier. The precipitated RNA was dissolved in 1× SSC and dialyzed for 48 h against 1× SSC at 4 °C. About 50–70% of the acid-insoluble radioactivity of the incubation mixture was recovered in the isolated RNA. This in vitro synthesized radioactive RNA, after 60 min of incubation, was hybridized to the pea ctDNA restricted by endonucleases *Sal*I, *Sma*I, and *Sal*I/*Sma*I. The autoradiographs of the Southern hybrids are shown in Figure 2. From this figure, it is clearly seen that the in

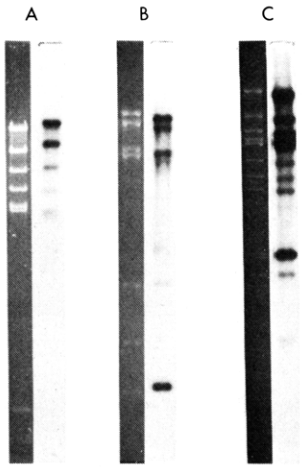

FIGURE 2: Hybridization of in vitro synthesized RNA with Southern blots of *Sal*I (A), *Sma*I (B), and *Sal*I/*Sma*I digests. Photographs of ethidium bromide fluorescence are shown alongside autoradiographs of corresponding hybrids.

vitro synthesized RNA hybridized with practically all the DNA fragments produced by endonucleases. When the hybridization is carried out by the RNA synthesized only for 5 min, the most prominent bands in *Xba*I digest were those of fragments 2 and 7 (data not shown). The ribosomal RNA genes in *Xba*I digest of pea ctDNA were shown to be present in *Xba*I fragment 2. Similar hybridizations with *Hin*dIII digests show that the major bands hybridizing with the in vitro synthesized RNA were fragments of molecular sizes 1.17, 1.23, 1.34, and 6.9 kilobase pairs (kbp), which were found to contain rRNA gene. Even though there were strong regions of hybridization with the rRNA genes, other DNA fragments also were found to hybridize with the in vitro synthesized RNA. When hybridizations were carried out by the in vitro RNA synthesized after 60 min of incubation, autoradiographs showed that practically all of the DNA fragments produced by restriction endonucleases exhibited strong hybridization. Thus, the transcription complex from pea chloroplasts does not selectively transcribe certain regions of the DNA. In short-term incubation, there certainly are some regions of DNA that are relatively more transcribed than others; but as the time of transcription is increased, all the DNA regions are transcribed. These results are in contrast with those obtained with the transcription complex in *Euglena* (Hallick et al., 1979). Using the transcription complex of *Euglena*, these investigators reported that in vitro synthesized RNA showed strong hybridization with only four of the 28 *Eco*RI fragments: B, F, L, and P. *Eco*RI fragments F, L, and P have been found to contain ribosomal RNA genes. In similar experiments, in vitro synthesized RNA was found to hybridize predominantly with fragments *Bal* G and H, *Xho*I d, and *Bam*I D and E, all from the rRNA region.

Solubilization of RNA Polymerase. As described previously, RNA polymerase in the 65000*g* pellet obtained from the Triton X-100 disrupted chloroplasts did not respond to the exogenous DNA. All our attempts, using various detergents, temperatures, and exogenous nucleases to remove the endogenous DNA and solubilize the enzyme, were ineffective. In our studies of the enzymatic makeup of chloroplasts, we identified the presence of both endonuclease and exonuclease activity in Triton-disrupted chloroplasts (R. L. McKown and K. K. Tewari, unpublished results). These activities also were found to be associated with the 65000*g* pellet. Therefore, the 65000*g* pellet was taken up in TG buffer; the endogenous DNA was digested with the endogenous endo- and exo-

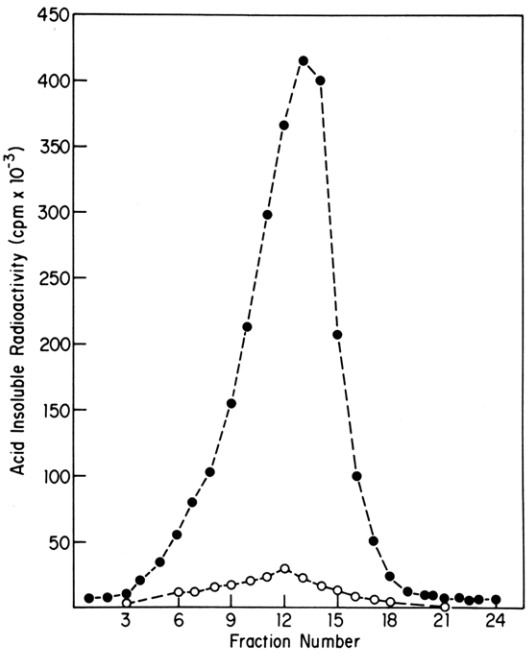

FIGURE 3: Purification of RNA polymerase in discontinuous glycerol gradients. 2 mL of the solubilized enzyme was layered on a discontinuous gradient of 70 (2 mL), 60 (2 mL), 55 (2 mL), 50 (2 mL), and 40% (2 mL) glycerol in 50 mM Tris-HCl, pH 8.5, 10 mM mercaptoethanol, and 0.2 mM PMSF. The gradients were centrifuged for 36 h in an SW41 rotor at 40 000 rpm. The gradient was fractionated and each fraction was assayed in the presence and absence of denatured DNA (10 μg). 50-μL aliquots were used in the regular assay mixture. (○) RNA polymerase activity without exogenous DNA; (●) RNA polymerase activity with *added* exogenous DNA.

nuclease(s) by incubating the suspension at 4 °C for 2–3 days. The solution then was centrifuged at 30000*g* for 30 min, and the supernatant was found to contain 90% of the 65000*g* pellet's polymerase activity. This supernatant was centrifuged for 36 h in a discontinuous gradient of 70, 60, 50, 45, and 40% glycerol in the SW41 rotor. Gradients were fractionated in 0.5-mL amounts, and the RNA polymerase activity was assayed with and without added DNA. The data are shown in Figure 3; RNA polymerase activity from the glycerol gradient is now totally dependent upon exogenous DNA. At the peak of activity, stimulation by adding exogenous DNA is about 15-fold (e.g., without DNA 30 000 cpm; with pea ctDNA 436 000 cpm). At this stage of purification, the pea chloroplast polymerase was about 3-fold more active in the presence of ctDNA compared to calf thymus DNA.

Purification of RNA Polymerase. During the purification steps, it became apparent that the RNA polymerase from chloroplast was extremely sensitive to salt concentration (Figure 4). At 25 mM K_2HPO_4, RNA polymerase activity was inhibited by 50%. When the concentration of potassium phosphate was raised to 50 mM, about 80% of the enzymatic activity was lost. In the presence of $(NH_4)_2SO_4$, the enzyme was again inhibited, and activity was reduced by 25% at 50 mM concentration. Of all the salts tested, NaCl showed the least inhibitory effect. Only 50% of RNA polymerase activity was inhibited at a salt concentration of 200 mM. But in the presence of 50 mM NaCl, RNA polymerase activity was not significantly affected. These experiments were carried out with solubilized enzyme, and salt was added during incubation. In order to find out whether the presence of salt permanently affected the enzyme or inhibition was observed only when salt was present during the assay, it was necessary to carry out the experiments by incubating the enzyme at 4 °C in the presence of 100 mM salt for different time intervals and then assaying

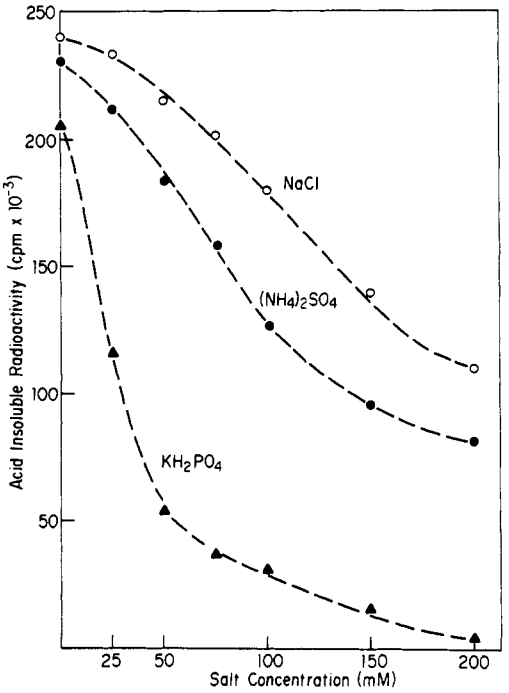

FIGURE 4: Effect of salt on RNA polymerase activity. 50 μL of the solubilized enzyme from the glycerol gradient was assayed in the presence of different concentrations of salt. The assay mixture was the same as described under Materials and Methods.

the enzyme after dialysis. In the presence of 100 mM K_2HPO_4, 50% of the activity was lost in 24 h, and no activity was detected after 48 h. Similarly, in the presence of 100 mM NaCl, there was a loss of 75% RNA polymerase activity after 48 h.

The extreme sensitivity of the enzyme to the presence of salt necessitated minimum exposure of the enzyme to salt concentrations and the use of only those methods that did not require fractionation by salt. Even though the solubilized enzyme was dependent upon exogenous DNA, it still was possible that the enzyme could be contaminated with small fragments of DNA. In order to completely remove the bound DNA, it was necessary to pass the solubilized enzyme through a DEAE-cellulose column equilibrated with TG buffer. All of the activity was bound to the DEAE-cellulose column (Figure 5). After the column was washed with 50 mM NaCl in TG buffer, the activity was eluted with a continuous NaCl gradient of 50–300 mM. The enzyme was found to elute at about 0.2 M, but the elution profile was quite broad. All of the RNA polymerase loaded onto the column was recovered. Activity was totally dependent upon addition of exogenous DNA. The pooled fractions quickly were dialyzed against TG buffer and concentrated with an Amicon filter 300. About 80% of activity from the glycerol gradient was recovered in the dialyzed fraction.

The RNA polymerase eluted from the DEAE-cellulose column was passed through phosphocellulose, calf thymus DNA–agarose, and native calf thymus DNA–cellulose. None of these compounds retained the RNA polymerase activity. For further purification, the enzyme was passed through Sepharose 6B equilibrated in TG buffer. The RNA polymerase was eluted as a single, sharp peak before elution of β-galactosidase (M_r 500 000). There was almost complete recovery of the enzyme, which showed about 2-fold purification at this step. Fractions containing the enzymatic activity were pooled, concentrated, and loaded on a 125-mL isoelectric focusing column.

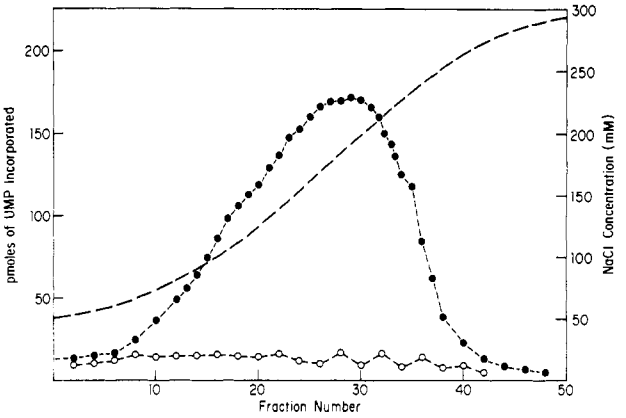

FIGURE 5: DEAE-cellulose chromatography of RNA polymerase. The RNA polymerase from the step glycerol gradient was directly loaded on to a 5-mL DE-52 column equilibrated in TG buffer. All of the enzymatic activity was bound to the column. The column was washed with 50 mM NaCl in TG buffer and eluted with a 50–300 mM linear NaCl gradient. (●) RNA polymerase activity with exogenous DNA; (○) RNA polymerase activity without exogenous DNA.

The RNA polymerase and other proteins associated with the enzyme were found to precipitate at an isoelectric point of 4.8. Isoelectric focusing also was carried out in the presence of 2.5% Triton; and proteins were found to still precipitate at their isoelectric points. Enzymatic activity was detected in the precipitated fractions. When the precipitated proteins were dialyzed against TG buffer, all of the enzymatic activity was recovered, and the precipitate was found to dissolve. However, there was no purification of the RNA polymerase by carrying out isoelectric focusing.

Fractions containing the RNA polymerase activity were combined, dialyzed extensively against TG buffer, concentrated, and fractionated on a 25–50% linear glycerol gradient. Fractions containing the RNA polymerase activity were analyzed for specific activity of the enzyme and their polypeptide composition.

Polypeptide Composition. Polypeptide composition of the transcribing complex solubilized by suspending it in TG buffer was analyzed by sodium dodecyl sulfate–polyacrylamide gels (Figure 7). There were 30–40 polypeptides ranging from molecular weights of 17 000 to 180 000. The most prominent band in this fraction was at about 55 kdaltons, which was identified as belonging to the large subunit of ribulosebisphosphate carboxylase. This fraction was found to contain polypeptides of 180, 140, 110, and 95 kdaltons, which were identified to be part of the RNA polymerase from corn leaves (Smith & Bogorad, 1974). When the transcription complex was self-digested with endogenous nuclease and the solubilized proteins were again analyzed, all the polypeptides bound to ctDNA were found to be released. Further purification of the enzyme through step glycerol gradients, DEAE-cellulose, and Sepharose 6B progressively removed most of the polypeptides of molecular size smaller than 27 kdaltons. $NaDodSO_4$ gel electrophoresis of fraction 26 from the continuous glycerol gradient (Figure 6) displaying maximum activity is shown in Figure 7. The most prominent polypeptides in this fraction were of 180, 140, 110, 95, 85, 80, and 65 kdaltons. There were also two smaller bands of 47 and 27 kdaltons.

When fraction 24 from continuous glycerol gradients (Figure 6) was analyzed, there were two dominant bands of 85 and 80 kdaltons and a few other lower bands. Since this fraction had practically no enzymatic activity, it was tentatively concluded that polypeptides of 180, 140, 110, 95, 65, 47, and 27 kdaltons probably constitute the RNA polymerase.

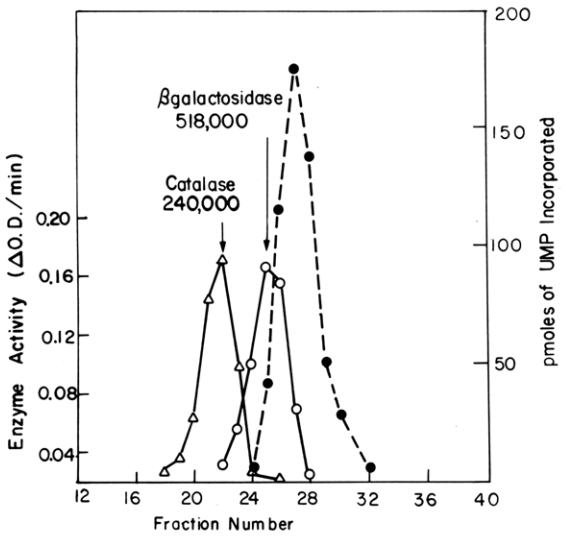

FIGURE 6: Fractionation of RNA polymerase in continuous glycerol gradient. Fraction 6 from the isoelectric focusing column was loaded on a 25–50% continuous glycerol gradient buffered with 50 mM Tris-HCl, pH 8.5, 10 mM mercaptoethanol, and 0.2 mM PMSF. The gradient was centrifuged for 36 h in a SW41 rotor at 40 000 rpm. In a separate tube, catalase and β-galactosidase were fractionated in a similar gradient.

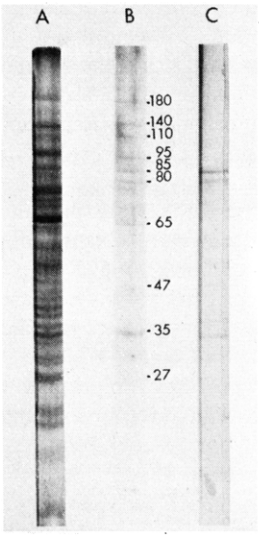

FIGURE 7: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns: (A) fraction 2 (Table I); (B) fraction 26 (Figure 6); (C) fraction 24 (Figure 6). Electrophoresis was carried out according to Weber & Osborn (1969).

The native molecular size of the RNA polymerase was determined by fractionation on a continuous glycerol gradient (Figure 6) in the presence of catalase and β-galactosidase. The molecular weight of the native polymerase was calculated to be over 500 000. It was not possible to exactly determine the molecular size of the enzyme at this stage because we could not rule out possible aggregation of proteins or the presence of small amounts of nucleotides. However, the banding pattern of the enzyme did not undergo any change when the gradients were run in the presence of 0.5 NaCl or the enzyme was treated with both DNase and RNase before centrifugation. The enzyme also was fractionated in a native gel electrophoresis. Figure 8 shows the fractionation of a step glycerol gradient purified fraction (fraction 3, Table I) and the most purified fraction 6 (Table I). The slowest moving protein was found to contain enzymatic activity. When native markers were run along side the enzyme, the RNA polymerase was found to again be much slower than β-galactosidase. Na-

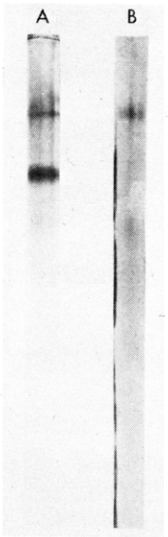

FIGURE 8: Fractionation of chloroplast RNA polymerase in 4% polyacrylamide under nondenaturing conditions: (A) fraction 3 (Table I); (B) fraction 7 (Table I).

Table I: Purification of RNA Polymerase

fraction no.	description	act. (pmol)	protein (mg)	sp act. (pmol/ mg of protein)	x-fold purification
1	Triton-disrupted chloroplasts	12400	545	13	
2	65000g pellet	8200	7.4	630	50
3	step glycerol	6300	2.2	2900	220
4	DEAE-cellulose	5700	1.8	3200	240
5	Sepharose 6B	6100	0.9	6800	520
6	isoelectric focusing	5300	0.75	7100	540
7	continuous glycerol gradient	4100	0.21	19520	1500

$DodSO_4$ electrophoresis of the gel fractions containing the RNA polymerase essentially revealed the polypeptide pattern obtained in Figure 7. Again, we were unable to definitely state polypeptide composition of the RNA polymerase because native gel electrophoresis is not specific in separating native proteins by their molecular sizes.

Properties of the Enzyme. The purified enzyme was found to show stronger preference for denatured DNA than for native DNA. When native λ DNA was used as a template, there was practically no enzymatic activity. The denatured λ DNA was found to be an excellent template. With denatured DNA, there was no obvious preference for ctDNA. The enzyme had an absolute requirement for divalent cations. Five millimolar Mg^{2+} was found to be optimal, but the enzyme was equally active if the $[Mg^{2+}]$ was raised to 15 mM. The most significant property of the enzyme was its inhibition by various salt concentrations as shown in Figure 4. The enzyme was inhibited neither by rifampicin (25 μg in 0.1 mL of assay mixture) nor by amanitine (5 μg in 0.1 mL of assay mixture). The pH optimum of the enzyme was found to be about 8.5, but the enzyme was quite active at a more alkaline pH (Figure 9). However, the enzyme was quite sensitive to acidic pH. The temperature optimum for the enzyme was about 30 °C (Figure 10) with a steep decline over 37 °C. At 50 °C, there was only 20% of the enzymatic activity found at 30 °C. This property of the pea chloroplast polymerase differs from that of the RNA polymerase from corn that was found to have a

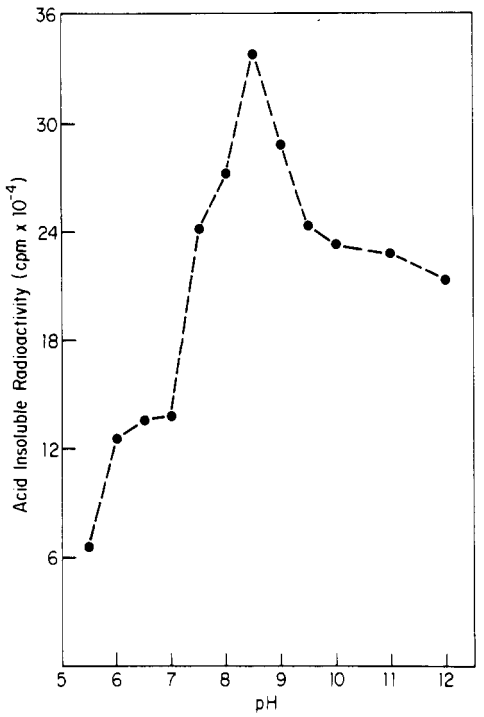

FIGURE 9: pH optimum of pea chloroplast RNA polymerase. From fraction 5 of Table I.

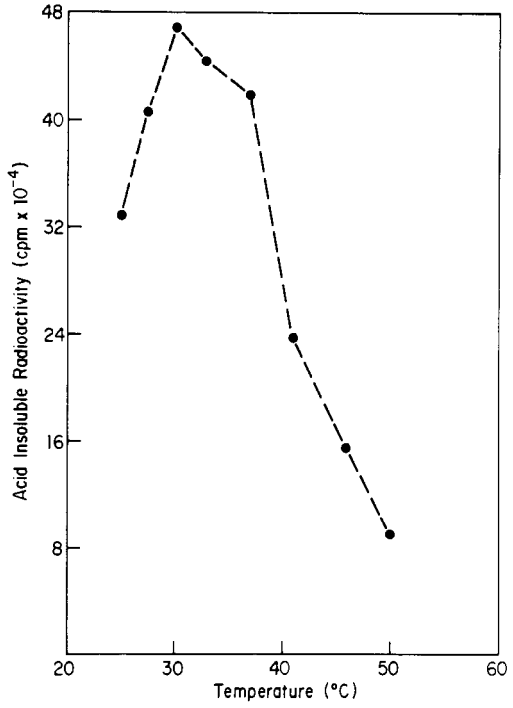

FIGURE 10: Temperature optimum of activity of pea chloroplast RNA polymerase. From fraction 7 of Table I.

temperature optimum of about 48 °C (Bottomley et al., 1971).

Discussion

DNA-dependent RNA polymerase from pea chloroplast is tightly bound to the membrane and was obtained in a complex with about 200-fold purification of the RNA polymerase activity. The complex obtained in fraction 2 (Table I), without first digesting the endogenous ctDNA, also was found to contain DNA polymerase activity along with endogenous nuclease activity. This complex was solubilized by adding TG buffer, pH 8.5, in the absence of Mg^{2+}. The solubilized RNA polymerase still contained endogenous DNA. The hybridization of in vitro synthesized RNA with restriction digests of ctDNA showed that practically all of the ctDNA was involved in transcription. Data of Southern hybrids showed strong hybridization with rRNA gene regions, but there were other areas of the genome that also showed strong hybridization. The pattern of hybridization obtained with the in vitro synthesized RNA was very similar to that obtained with polysomal RNA. However, we have not yet analyzed whether rRNA genes or mRNA genes (e.g., carboxylase) are faithfully transcribed by this complex in vitro.

Separation of the RNA polymerase from the endogenous DNA was achieved by solubilizing the RNA polymerase in TG buffer, pH 8.5, in the presence of 1 mM Mg^{2+} at 4 °C. At low levels of Mg^{2+}, endogenous and exogenous nucleases of chloroplasts were able to digest the endogenous DNA. We have routinely incubated the Triton-disrupted chloroplasts for 72 h at 4 °C. We have also tried to reduce the time to digest endogenous DNA by increasing the temperature of incubation. It was possible to remove endogenous DNA at a shorter time, but the RNA polymerase activity was found not to be stable at higher temperatures. After removal of the endogenous DNA, the enzyme could be fractionated on glycerol gradient or on DEAE-cellulose. Purification of enzymatic activity was 1500-fold, starting from the Triton-disrupted chloroplasts. The specific activity of the most purified fraction was about 20 nmol of UMP incorporated per mg of protein per 30 min. This specific activity compares well with the specific activity of 25–35 nmol of AMP incorporated per mg of protein per 20 min for the ctRNA polymerase from corn chloroplasts (Bottomley et al., 1971). In contrast, *Escherichia coli* RNA polymerase was reported to have a specific activity of 450 nmol of AMP incorporated per mg of protein per 10 min.

The molecular size of pea RNA polymerase was estimated to be well over 500 kdaltons by centrifugation in glycerol gradients, fractionation on a Sepharose 6B column, and native gel electrophoresis. Unfortunately, none of these methods can accurately determine the molecular size of native enzyme. The peak fraction from continuous glycerol gradients was found to contain nine polypeptides of 180, 140, 110, 95, 80, 65, 47, and 27 kdaltons. Of these, polypeptides of 85 and 80 kdaltons also were found to be present in fractions where there was little enzymatic activity. From these data, one could infer that pea ctRNA polymerase contains polypeptides of 180, 140, 110, 95, 65, 47, and 27 kdaltons. The sum of molecular weights of these polypeptides would explain the molecular size of the native RNA polymerase. However, our enzyme is not yet completely pure enough to make such conclusions. The molecular sizes of the polypeptides of pea chloroplast RNA polymerase are remarkably close to those obtained for RNA polymerase from maize chloroplasts (Smith & Bogorad, 1974; Jolly & Borogard, 1980). Briat & Mache (1980) have reported the isolation of RNA polymerase from spinach that contains five polypeptides of 69, 60, 55, 34, and 15 kdaltons. It is difficult to interpret their data because the RNA polymerase has not been highly purified and the native gel electrophoresis of the enzyme is carried out at room temperature. Under similar experimental conditions, pea RNA polymerase is completely inactivated.

The solubilized and purified RNA polymerase from pea chloroplasts is not inhibited by rifampicin or amanitine, a unique property of chloroplast RNA polymerase that also has been found to be true for the RNA polymerase of corn (Bottomley et al., 1971) and *Euglena* (Hallick et al., 1976). We do not know whether there are two different RNA po-

lymerases in chloroplasts. If the two polymerases contain polypeptides of close molecular weights, the analytical tools described in this paper would not distinguish them. The solubilization and purification of RNA polymerase from chloroplasts has provided us with a method to study the detailed transcription mechanism of the chloroplast genome.

Registry No. RNA polymerase, 9014-24-8.

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Granular Pneumocytes in Primary Culture Secrete Several Major Components of the Extracellular Matrix†

Helene Sage,* Federico M. Farin, Gary E. Striker, and Aron B. Fisher

ABSTRACT: Primary cultures of rat alveolar type II epithelial cells (granular pneumocytes) produced several components of the pulmonary extracellular matrix. Fractionation by ion-exchange chromatography of radiolabeled protein secreted into the culture medium resulted in the partial purification of two of these components: fibronectin and type IV procollagen. Identification of these proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was confirmed by radioimmune-precipitation studies with affinity-purified antibodies. Thrombospondin, a platelet α-granule protein that was recently shown to be secreted by endothelial and other mesenchymally derived cells and may be involved in platelet aggregation, was, in addition, purified by elution from diethylaminoethylcellulose with 0.5 M NaCl. The levels of these secreted proteins were measured by radioimmune precipitation. Of the total radiolabeled culture medium protein secreted during a 24-h period by the granular pneumocytes, fibronectin, type IV procollagen, and thrombospondin represented 3–15%, 2%, and 3%, respectively. The biosynthesis, by alveolar epithelial cells, of proteins that constitute or are closely associated with the alveolar basement membrane implies that this structure is at least partially derived from the cells themselves. Furthermore, it suggests that the type II epithelial cell is involved in pulmonary cytodifferentiation, in lung morphogenesis and repair, and in certain interstitial lung disorders in which derangement of the extracellular matrix occurs.

More than 40 different cell types have been described in adult human lung [for a review, see Kuhn (1976)]. This cellular heterogeneity reflects not only the structural diversity of the lung architecture in toto but also the metabolic and functional properties of this organ as well. In recent years, an increased understanding of the primary gas-exchanging unit, the pulmonary alveolus, has been achieved by approaches at the cellular level. Studies dealing with alterations in cell populations and connective tissue proteins in the interstitial lung disorders have, in particular, established that an important relationship exists between alveolar function and its cellular/extracellular matrix composition [for a review, see Crystal et al. (1978)].

The normal adult alveolus contains two major cellular populations: parenchymal and inflammatory/immune effector cells. The latter group is comprised mainly of macrophages (80–90%) and lymphocytes (Crystal et al., 1978). There are four major types of alveolar parenchymal cells. Interstitial mesenchymal connective tissue ("fibroblast-like") cells are found principally in alveolar septae and comprise approximately 39% of the total parenchymal cell population (Kuhn, 1976; Crystal et al., 1978). The pulmonary alveolus is lined with type I and type II epithelial cells, representing 8% and 14% of the parenchymal cell types, with the remainder contributed by capillary endothelial cells (Crystal et al., 1978).

† From the Departments of Biochemistry (H.S.) and Pathology (F.M.F. and G.E.S.), School of Medicine, University of Washington, Seattle, Washington 98195, and the Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 (A.B.F.). *Received December 10, 1982.* This work was supported in part by National Institutes of Health Grants HL 18645, AM 11248, HL 27218, GM 29853, GM 07187, GM 21797, and HL 19737 and by Juvenile Diabetes Foundation Grant 79R134. H.S. is an Established Investigator of the American Heart Association, with funding contributed in part by the Washington Heart Association.

* Address correspondence to this author at the Department of Biological Structure, School of Medicine, University of Washington, Seattle, WA 98195.

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