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RNA Polymerases of Maize. Purification and Molecular Structure of DNA-dependent RNA Polymerase II*

(polypeptide subunits)

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ABSTRACT **Nuclear DNA-dependent RNA polymerase II has been purified from leaves of *Zea mays* by a new procedure that improves enzyme stability and thus permits more manipulation during purification. The purification procedure includes a heating step, gel filtration on Sepharose 6B and 4B, and chromatography on DEAE- and DNA-celluloses. This method of purification yields an enzyme that exhibits maximal activity when denatured DNA is used as a template. Electrophoresis of highly purified enzyme on polyacrylamide gels containing sodium dodecyl sulfate indicates that maize RNA polymerase IIa is composed of several polypeptide subunits. The most highly purified preparations contain polypeptides with molecular weights of 200,000, 160,000, 35,000, 25,000, 20,000, and 17,000.**

Multiple forms of DNA-dependent RNA polymerase have been identified in nuclei of eukaryotic cells (1). Enzyme I is believed to be of nucleolar origin and, thus, to be involved in ribosomal RNA synthesis while Enzyme II is located in the nucleoplasm and is involved in synthesis of DNA-like RNA (2-4). It was shown earlier that two analogous RNA polymerases are present in nuclei of *Zea mays* (5). Furthermore maize Enzyme II was resolved upon chromatography on DEAE-cellulose into two activities: one (IIb) more active with native and the other (IIa) more active with denatured nuclear DNA of maize. Conditions for conversion of IIb into IIa were also described. An RNA polymerase with distinctly different properties has been prepared from maize chloroplasts (6).

Several groups (7-9) have reported on the purification and molecular subunit structure of polymerase II from various animal sources. Weaver *et al.* (10) have suggested that RNA polymerase II of calf thymus is composed of four polypeptide chains of molecular weights 190,000, 150,000, 35,000, and 25,000. Kedinger *et al.* (11, 12) have also reported that nuclear RNA polymerases of calf-thymus are comprised of several different polypeptides. We report here a new purification procedure for maize enzyme IIa and present evidence that it is composed of several polypeptide chains.

METHODS

Preparation of DNA-dependent RNA polymerase IIa

Filtered homogenates of maize (*Zea mays*, WF 9 TMS x B37, Illinois Foundation Seeds, Inc., Champaign, Ill.) leaves were clarified by high-speed centrifugation as described (5). In a typical preparation, 4 kg of fresh weight of corn leaves yielded 5600 ml of clear amber supernatant (Fraction 1) after centrifugation of leaf homogenate at 100,000 × *g* for 90 min. The protein fraction that precipitated from this solution between 30 and 50% saturation of $(NH_4)_2SO_4$ was collected, dissolved in 150 ml of 50 mM Tris·HCl (pH 8.0)-20% glycerol-10mM 2-mercaptoethanol (TGM) buffer and dialyzed against several changes of the same buffer. The solution was adjusted to a protein concentration of 25 mg/ml by addition of TGM (Fraction 2). It was heated to 50° for 10 min, after which it was chilled and centrifuged at 50,000 × *g* for 20 min in a Type 30 rotor in a Spinco preparative ultracentrifuge; the supernatant was collected (Fraction 3).

Sepharose Chromatography. Fraction 3 (130 ml) was applied to a 5.0 × 140-cm column of Sepharose 6B (Pharmacia Fine Chemicals) equilibrated with TGM buffer. The column was washed with the same buffer at a flow rate of 90 ml/hr (Fig. 1). The tubes containing polymerase activity (e.g., fractions 51-70, Fig. 1) were pooled and concentrated by bringing the solution to 50% of saturation with $(NH_4)_2SO_4$. The pellet was dissolved in 30 ml of TGM buffer and dialyzed against the same buffer (Fraction 4). The solution was then applied to a 5 × 100-cm Sepharose 4B (Pharmacia Fine Chemicals) column from which the enzyme was eluted with TGM buffer at a flow rate of 25 ml/hr. The active fractions were pooled (e.g., fractions 75-95, Fig. 2) to give Fraction 5 (Table 1).

DEAE-Cellulose Chromatography. Fraction 5 was applied to a DEAE-cellulose (Whatman DE-52) column prepared and equilibrated according to Burgess (13). RNA polymerase was eluted with a gradient of $(NH_4)_2SO_4$ in TGM buffer (Fig. 3). The active fractions were pooled (Fraction 6).

Phase Partition Chromatography. Fraction 6 was further purified by phase partition chromatography according to Alberts (14). The phase containing RNA polymerase was exhaustively dialyzed against TGM buffer (Fraction 7).

DNA-Cellulose Chromatography. Fraction 7 was applied to a DNA-cellulose column containing single-stranded calf-thymus DNA prepared according to Alberts *et al.* (15) (Fig. 4).

Polyacrylamide-Gel Electrophoresis. Sodium dodecyl sulfate-containing gels were prepared and run according to Weber and Osborn (16). The 10-cm gels contained 0.1% Na dodecyl sulfate and either 5% acrylamide or an upper 5% plus a lower 10% portion. Polyacrylamide gels for electrophoresis under nondenaturing conditions were prepared to contain

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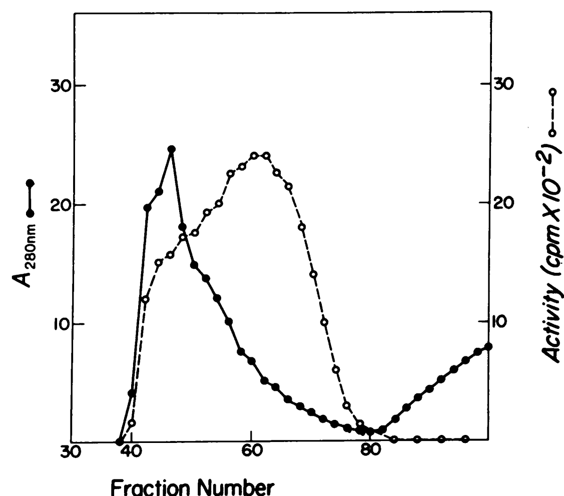

FIG. 1. Sepharose 6B column profile. Fraction *3* (130 ml, 3.3 g of protein) was applied to a 5 × 140-cm column of Sepharose 6B. Protein was eluted, and 20-ml fractions were collected. Aliquots of 50 μl per fraction were assayed. Fractions 51-70 were pooled (= Fraction *4*, Table 1).

4% acrylamide and were run at pH 8.7 according to the methods of Ornstein (17) and Davis (18). Gels were stained with 0.25% Coomasie Brilliant Blue R250 (Colab Laboratories, Inc.) in methanol-acetic acid-water (5:1:5) and were destained electrophoretically in 7.5% acetic acid-5% methanol.

Samples for Na dodecyl sulfate-gels were incubated in 1% Na dodecyl sulfate-1% 2-mercaptoethanol-10 mM phosphate buffer (pH 7.0) for 5 min at 90° and then for 30 min at 37°. Glycerol was added to about 40% before samples were applied to the gels. Dilute samples were dialyzed into 0.2 M acetic acid and frozen-dried before being dissolved in Na dodecyl sulfate solutions.

TABLE 1. *Purification of polymerase II*

Fraction	Specific activity*	Yield (%)
1. High-speed supernatant	0.035	100
2. Ammonium sulfate fraction (30-50%)	0.136	48
3. Ammonium sulfate fraction heated	0.216	36
4. Pooled Sepharose 6B peak	6.9	36
5. Pooled Sepharose 4B peak	7.6	29
6. Pooled DEAE-cellulose peak	24.9	15
7. Phase partition	24.9	10
8. Pooled DNA-cellulose peak	≈121†	5

The assay for enzymatic activity was described earlier (5). The assay mixture contained [^{14}C]ATP (Schwarz BioResearch); 53.2 Ci/mol. Protein concentrations were estimated by the method of Lowry *et al.* (19), with bovine-serum albumin (Calbiochem) as a standard.

* Specific activity is expressed as nM of [^{14}C]AMP incorporated per mg of protein per 20 min at 37°.

† The protein concentration of the pooled DNA-cellulose peads was determined by summing the intensities of all bands stained with Coomasie Blue, assuming a protein concentration of 10 μg per band.

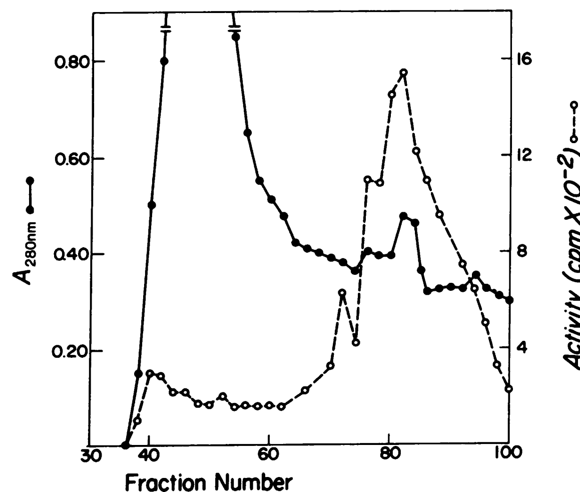

FIG. 2. Sepharose 4B column profile. Fraction *4* (30 ml, 114 mg of protein) was applied to a 5 × 100-cm column of Sepharose 4B. Protein was eluted, and 15-ml fractions were collected. Aliquots of 50 μl were assayed. Fractions 75-95 were pooled (= Fraction *5*, Table 1).

RESULTS AND DISCUSSION

Purification of maize RNA polymerase IIa

Maize nuclear RNA polymerases were prepared previously (5) by DEAE-cellulose chromatography of an $(NH_4)_2SO_4$ fraction. This procedure resulted in considerable purification of enzyme II, but further purification was impossible due to the extreme lability of the enzyme after ion-exchange chromatography. The improved procedure reported here (Table 1) differs from the one already published (5) by addition of the following steps: (*a*) heating of the 30-50% $(NH_4)_2SO_4$ fraction; (*b*) gel filtration with Sepharose 6B and 4B; (*c*) partition between polyethylene glycol and dextran phases (14); and (*d*) chromatography on DNA-cellulose columns.

The heating step in the procedure destroyed enzyme I activity (5) and, thus, this method is suitable only for preparation of enzyme II. Heating the 30-50% $(NH_4)_2SO_4$ fraction to 50° for 10 min resulted in precipitation of 50% of the protein. However, the supernatant, recovered by centrifugation, contained 75% of the RNA polymerase activity

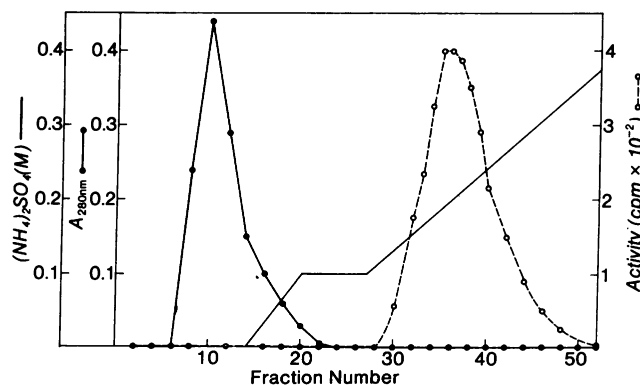

FIG. 3. DEAE-cellulose column profile. Fraction *5* (8.6 mg of protein) was applied to a 6.0 × 7.5-cm column of DEAE-cellulose and eluted as described (5). The enzyme activity was eluted at 0.18 M $(NH_4)_2SO_4$. Aliquots of 20 μl per fraction (10 ml) were assayed. Fractions 30-47 were pooled (= Fraction *6*, Table 1).

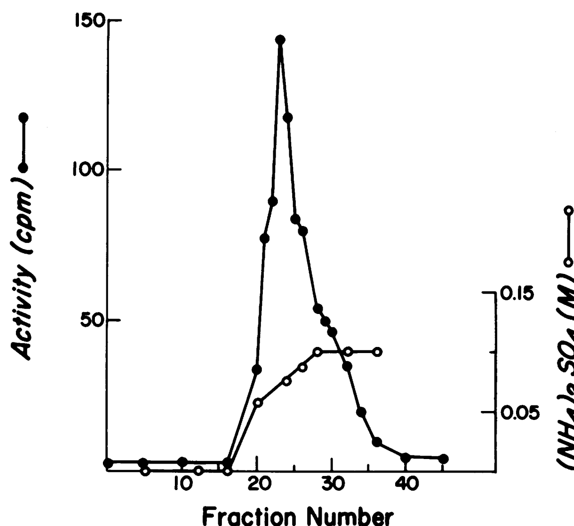

FIG. 4. DNA-cellulose column profile. Fraction *7* (2 mg of protein) was applied to a 1 × 1.5-cm column of DNA-cellulose and eluted. Fractions of 1.0 ml were collected, and aliquots of 50 µl per fraction were assayed. Fractions 20-30 were pooled (= Fraction *8*, Table 1).

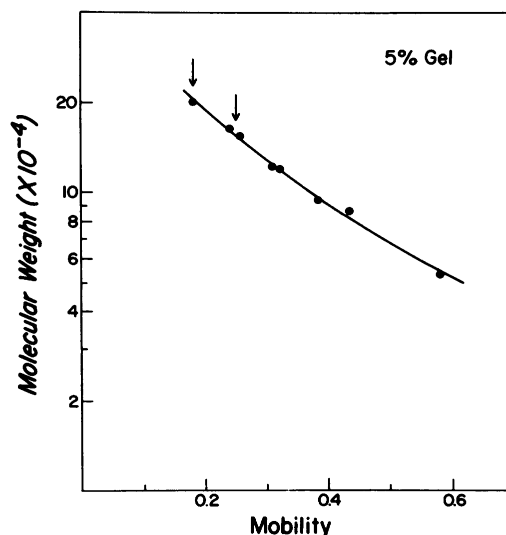

FIG. 6. Mobility of polypeptides against log molecular weight on 5% acrylamide gels. *Arrows:* large subunits of maize RNA polymerase IIa. Marker proteins (13, 16), *circles:* glutamate dehydrogenase (53,000); ovalbumin dimer (86,000); phosphorylase a (94,000); β-galactosidase (135,000); bovine-serum albumin dimer (136,000); *E. coli* RNA polymerase β and β' subunits (155,000 and 165,000); myosin (200,000).

present in the 30–50% ammonium sulfate fraction before heating.

Polymerase IIa was eluted from Sepharose 6B as a broad peak (Fig. 1). Although the minimum molecular weight of the enzyme is about 450,000, it behaved as a much larger entity on the Sepharose 6B column (the exclusion size of Sepharose 6B is 4×10^6 for globular proteins); this could occur if the enzyme were attached to DNA pieces of different sizes at this stage. This step provides a quick and excellent purification (32-fold). Furthermore, the enzyme preparation (Fraction 4) is stable for several days at 4° after gel filtration if the protein concentration is kept above 1 mg/ml. As mentioned in an earlier report (5), the 30–50% $(NH_4)_2SO_4$ fraction (Fraction 2) transcribed native DNA more efficiently than denatured DNA. However, after chromatography on Sepharose 6B, polymerase II prefers denatured DNA and, thus, is mainly or entirely in the IIa form. This alteration of specificity is analogous to the situation described previously (5) in which centrifugation of Fraction 2 on glycerol gradients yielded an activity that functioned better on denatured than native DNA. The change in template specificity after a size-discriminating step observed in the present experiment might reflect the loss of a specific factor necessary for reading native DNA.

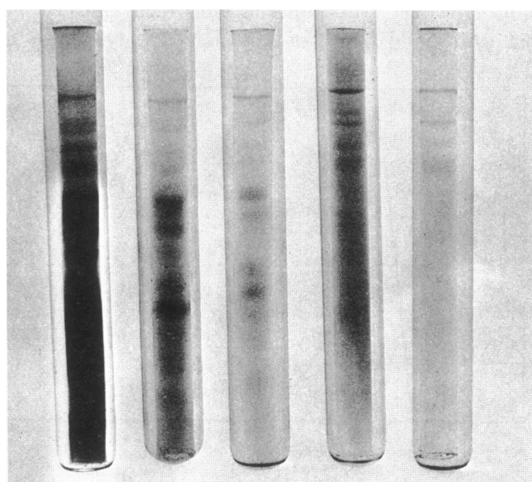

FIG. 5. Na dodecyl sulfate-5% acrylamide gel electrophoresis at each stage of purification of enzyme IIa. The gels represent the following steps (*left* to *right*): (*1*) 30-50% ammonium sulfate fraction; (*2*) Sepharose 6B; (*3*) Sepharose 4B; (*4*) DEAE-cellulose; (*5*) DNA-cellulose.

Subsequent chromatography on Sepharose 4B allowed the separation of RNA polymerase from the bulk of the remaining proteins (Fig. 2).

Upon chromatography on DEAE-cellulose, the enzymatic activity was eluted at 0.20 M $(NH_4)_2SO_4$ as a symmetrical peak (Fig. 3). This peak corresponds to activity IIa described in an earlier report (5).

This material did not bind predictably when applied to a DNA-cellulose column. Although at this stage of purification activity is completely dependent upon addition of exogenous DNA to the assay mixture in order to synthesize trichloroacetic acid-precipitable RNA, it seemed possible that small pieces of DNA were still adhering to the enzyme and preventing binding to DNA-cellulose. After the enzyme was partitioned into polyethylene glycol (nucleic acids were found in the dextran phase), all of the enzyme bound to DNA-cellulose. It was eluted as a single peak at 0.08 M $(NH_4)_2SO_4$ (Fig. 4).

Polyeptide chain composition of the enzyme

At each stage of purification, aliquots of fractions containing polymerase activity were analyzed by electrophoresis on 5% polyacrylamide gels containing 0.1% Na dodecyl sulfate (Fig. 5). This analysis revealed that low-molecular-weight polypeptides are eliminated by chromatography on Sepharose 6B and 4B (compare tubes *1* with *2* and *3*, Fig. 5) and that the active fractions eluted from DEAE-cellulose columns (tube

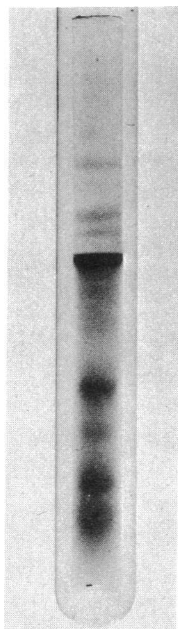

FIG. 7. Na dodecyl sulfate-acrylamide (5 and 10%) gel electrophoresis of enzyme IIa after DNA-cellulose chromatography.

4, Fig. 5) contain three major polypeptides of high molecular weight (200,000, 170,000, and 160,000) and several less abundant polypeptides of lower molecular weight. Electrophoresis of enzyme purified by DNA-cellulose on 5% polyacrylamide gels containing Na dodecyl sulfate revealed two major polypeptides of molecular weight 200,000 and 160,000 and some lower molecular-weight-polypeptides in bands too diffuse to locate with precision (tube *5*, Fig. 5).

Molecular weights were computed by comparison of mobility of polypeptides in RNA polymerase preparations with mobilities of proteins of known molecular weights (Fig. 6) (16). Molecular weight estimations of the larger subunits are subject to more uncertainty (±10%) due to the lack of a strictly linear relationship between mobility and log molecular weight for polypeptides over 130,000 (13).

In order to further resolve the polypeptides in RNA polymerase preparations, we used "mixed gels" according to the method of Gissinger and Chambon (20). This technique involves using a lower half containing 10% acrylamide, in which lower-molecular-weight components are resolved, and an upper half containing 5% acrylamide, in which higher-molecular-weight components are resolved. Electrophoresis of enzyme purified by DNA-cellulose reveals the pattern shown in Fig. 7, in which polypeptides of molecular weight 200,000, 160,000, 100,000, 80,000, 35,000, 25,000, 20,000, and 17,000 are visible. Fig. 8 shows the calibration curve for the 10% polyacrylamide portion of the gel; the positions of the four smaller polypeptides in the RNA polymerase IIa preparation are shown by *arrows*.

The amount of material of 200,000 molecular weight varies among different preparations of enzyme purified by DNA-cellulose. In most preparations, the 200,000- and 160,000-dalton polypeptides are present in about equimolar amounts, based on absorbance scans of stained gels and on the assumption that the two polypeptides absorb Coomassie Blue equally in proportion to their size. However, in the preparation in Fig. 7, the ratio of material at 200,000 to that at 160,000 was about 0.4. In instances where a decreased amount of 200,000-dalton material is observed, there is a concomitant appearance of polypeptides of molecular weight 100,000 and 80,000 (compare tube *5*, Fig. 5, with Fig. 7). We conclude that the polypeptides of 100,000 and 80,000 molecular weight are breakdown products of higher-molecular-weight polypeptides and are not part of active RNA polymerase IIa.

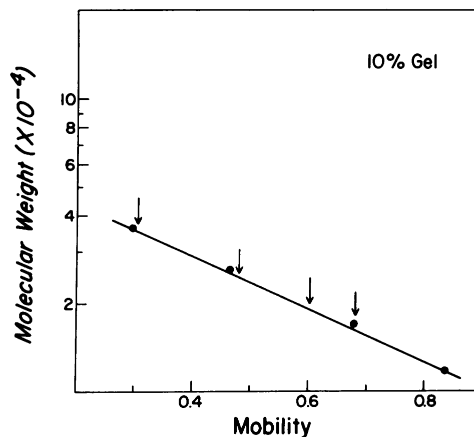

FIG. 8. Mobility of polypeptides against log molecular weight for 10% acrylamide gels. *Arrows:* RNA polymerase IIa. Marker proteins (16), *circles:* cytochrome *c* (11,700); myoglobin (17,200); α-chymotrypsin (25,700); lactate dehydrogenase (36,000).

To explore further whether the various polypeptides in our most highly purified preparations of RNA polymerase IIa are essential for activity, enzyme purified by DNA-cellulose was stored at 4° for 24 hr, a treatment that destroys activity. Gel electrophoresis of such a stored preparation on 5% gels reveals that the subunit of molecular weight 160,000 is

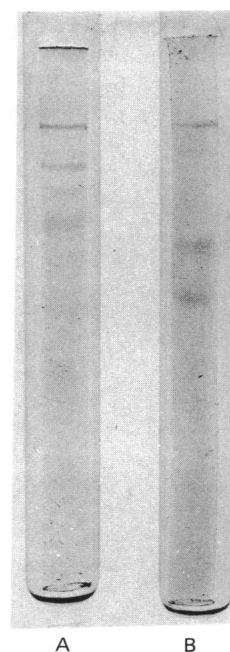

FIG. 9. Na dodecyl sulfate-5% acrylamide gel electrophoresis of enzyme IIa after DNA-cellulose chromatography. (*A*) Active enzyme; (*B*) inactive enzyme (stored at 4° for 24 hr).

absent but instead 90,000- and 70,000-dalton polypeptides occur (Fig. 9*B*). The latter two polypeptides are most probably breakdown products of the subunit of molecular weight 160,000. It appears that an intact 160,000-dalton polypeptide (Fig. 9*A*) is necessary for activity of maize RNA polymerase IIa.

Further evidence for the occurrence of the two large polypeptides together in RNA polymerase IIa was obtained by gel electrophoresis under nondenaturing conditions. Gels of 4% acrylamide contained one prominent band of protein and some material that did not enter the gel. The protein-containing bands from several gels were extracted in the cold (4°) with 10 mM phosphate and dialyzed into 0.2 M acetic acid. The dialyzed extract was concentrated by freeze-drying, dissolved in a solution of Na dodecyl sulfate and 2-mercaptoethanol, incubated, and then analyzed by electrophoresis on a 5% polyacrylamide–Na dodecyl sulfate gel. Two large polypeptides (molecular weights about 200,000 and 160,000) were detected; some smaller polypeptides were also present but in diffuse bands.

The remaining smaller polypeptides in RNA polymerase purified by DNA–cellulose (molecular weights 35,000, 25,000, 20,000, and 17,000) are regularly present in preparations of this purity, and thus it has not been possible to correlate absence of any of these polypeptides with loss of enzyme activity. These polypeptides are intact components of the active enzyme; or they could be either breakdown products of higher-molecular-weight subunits of the polymerase that remain associated with the enzyme complex or subunits of proteins that copurify with RNA polymerase.

Assuming that the polypeptides of molecular weight 100,000, 80,000, 90,000, and 70,000 seen in some preparations are artifacts of proteolysis as suggested and that the low-molecular-weight polypeptides resolved on the 10% acrylamide gel are intact components of the enzyme, the subunit composition of maize RNA polymerase IIa purified as described includes polypeptides of molecular weight 200,000, 160,000, 35,000, 25,000, 20,000, and 17,000. Such a subunit composition would yield a molecular weight of 457,000, which correlates well with results presented earlier (5). These results are somewhat similar to those reported for a calf-thymus enzyme by Kedinger *et al.* (11, 12). Their preparations of enzyme BI (about equivalent to our maize enzyme IIa) contained polypeptides of molecular weight 214,000, 140,000, 34,000, 25,000, and 16,500.

Highly purified preparations of maize chloroplast RNA polymerase described by Bottomley *et al.* (6) contain two large polypeptides of molecular weight 220,000 and 150,000 and several smaller polypeptides (H. J. Smith and L. Bogorad, in preparation).

NOTE ADDED IN PROOF

G. M. Polya [(1973) *Arch. Biochem. Biophys.* **155**, 125–135] has recently purified a soluble DNA-dependent RNA polymerase from wheat seedling leaves. The enzyme is insensitive to α-amanitin. It is composed of a single polypeptide with a molecular weight of 65,000. Polya suggests that it resembles the RNA polymerases of mitochondria from *Neurospora crassa* and rat liver with regard to subunit composition and aggregation characteristics.

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