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THE 180 KDa POLYPEPTIDE CONTAINS THE DNA-BINDING DOMAIN OF RNA POLYMERASE II

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SUMMARY: Purification of RNA polymerase II from chicken myeloblastosis (leukemia) cells to homogeneity and subsequent structural analysis of the purified enzyme revealed that the enzyme contained seven polypeptides with molecular masses ranging from 27 KDa to 220 KDa. Inclusion of protease inhibitors in the buffer system during purification significantly increased the molar ratio of the largest (220 KDa) polypeptide to the second largest (180 KDa) polypeptide. However, proteolytic conversion of the 220 KDa to 180 KDa polypeptide did not inhibit the DNA binding activity of the enzyme. The enzyme, after dissociation into subunits in a SDS-polyacrylamide gel containing urea, was blotted onto a nitrocellulose filter. The filter was incubated with ^{32}P-labeled calf thymus DNA and both the 220 KDa and 180 KDa polypeptides of the enzyme bind DNA, suggesting that the DNA-binding site of the enzyme resides on the 180 KDa polypeptide of the largest subunit.

RNA synthesis in eukaryotic cells is carried out by three distinct classes of RNA polymerase, designated I, II, and III; all three classes of polymerase reported so far are complex enzymes each consisting of six to twelve subunits (1-3). Because of the complexity of the enzymes, the instability of animal polymerases, and the relatively small amounts of pure enzymes which are available, a successful reconstitution experiment has not been reported. Therefore, little is known about the functions of the various subunits of eukaryotic RNA polymerases. Nevertheless, by using affinity labeling techniques it has been shown that the RNA polymerase II-specific inhibitor α-amanitin binds selectively to the B3 (145 KDa) subunit of the enzyme (4), and that the nucleoside triphosphate binding site resides on the 37 KDa subunit of RNA polymerase II (5). The largest (190 KDa) subunit of RNA polymerase I of mouse myeloma cells was reported to contain a catalytic center, which is involved in RNA chain elongation (6). The nucleotide sequence coded for the largest (220 KDa) subunit of RNA polymerase II of yeast (7) or Drosophila (8) has been determined and was shown to have sequence homologies

The abbreviations used are: SDS, sodium dodecyl sulfate; PMSF, phenylmethenesulfonyl fluoride.

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with the genes coded for the largest (β') subunit of E. coli RNA polymerase, implicating the involvement of the largest subunit of RNA polymerase II in DNA binding. Analysis of amino acid composition of the IIa (210-220 KDa) and IIb (170-180 KDa) subunits of RNA polymerase II suggested that IIa differs from IIb by having a C-terminal sequence rich in serine, proline, threonine, and tyrosine (9). In this study, we have extensively purified the RNA polymerase II from chicken myeloblastosis leukemia cells, analyzed the subunit structure, and demonstrated by direct enzyme-DNA interactions that the DNA-binding domain of the largest subunit of RNA polymerase II resides in the 180 KDa polypeptide.

METHODS

RNA polymerase purification: RNA polymerases of chicken myeloblastosis cells were solubilized from purified nuclei and the soluble nuclear RNA polymerases were subjected to DEAE-Sephadex column chromatography as described previously (10,11). Fractions of the column eluate containing RNA polymerase II activity were combined, diluted with TGMED buffer (0.05 M Tris-HCl, pH 7.9; 30% glycerol; 5 mM $MgCl_2$; 0.1 mM EDTA and 0.5 mM dithiothreitol) to 0.1 M with regard to ammonium sulfate concentration, and loaded onto a phosphocellulose column (2.7 x 5 cm) pre-equilibrated with 0.05 M ammonium sulfate in TGMED. The column was then washed with 50 ml of 0.1 M ammonium sulfate in TGMED. The flow through and wash fractions of phosphocellulose column were combined and loaded onto a heparin-Sepharose column (0.9 x 2.8 cm) equilibrated with 0.2 M ammonium sulfate in TGED buffer (0.05 M Tris-HCl, pH 7.9; 25% glycerol; 0.1 mM EDTA and 0.5 mM dithiothreitol). The column was washed with 0.25 M ammonium sulfate in TGED and the RNA polymerase was eluted with 0.8 M ammonium sulfate in TGED. Fractions were collected at 1 ml and 0.5 ml aliquots for the 0.25 M and 0.8 M elution, respectively. A 0.01 ml part of each fraction was assayed for RNA polymerase activity. The peak fractions of RNA polymerase II from the heparin-Sepharose column were pooled and brought to 85% saturation with ammonium sulfate by adding solid ammonium sulfate and subjected to 10-40% glycerol gradient sedimentation as previously described (10,11). Bovine serum albumin, E. coli RNA polymerase and chicken myeloblastosis DNA polymerase α (12) were run on parallel gradients to serve as markers. Fractions were collected from the bottom of gradients, and 5 µl aliquots were assayed for RNA polymerase activity.

RNA polymerase assay: The standard RNA polymerase assay system was as described (10,11). One unit of RNA polymerase activity is defined as 1 nmole of [^{3}H] UMP incorporated per 30 min. at 37 °C in a volume of 0.1 ml. Protein concentrations were determined according to Bradford (13).

Polyacrylamide gel electrophoresis: Chromatographically purified RNA polymerase II was subjected to electrophoresis on polyacrylamide gels in slabs under both denaturing (SDS-containing) and non-denaturing conditions (14.15). The proteins were stained overnight in 0.5% Coomassie Brilliant Blue R, 50% methanol, and 7.5% acetic acid; and destained in 45% methanol and 9% acetic acid. Molar ratios of RNA polymerase polypeptides were measured using Zeineh Soft Laser Scanning Densitometer (model SL-504-XL, Biomed Instruments, Inc.).

Protein blotting and DNA-binding: The procedures used to identify RNA polymerase subunits that bind DNA was similar to that described by Bowen et al. (16) for the detection of specific protein-DNA interactions. Briefly,

RNA polymerase II (12 µg) was subjected to electrophoresis in SDS-polyacrylamide gel (10% acrylamide) containing 4 M urea. After electrophoresis the gel was treated as described (16) to remove SDS. The renatured polypeptides were blotted to nitrocellulose filters and incubated with ^{32}P-labeled calf thymus DNA (11). After extensive washing, the filters were dried and exposed to Kodak XR-5 film to reveal the DNA-binding polypeptides.

RESULTS

Purification of chicken myeloblastosis RNA polymerase II: Nuclear RNA polymerases from chicken myeloblastosis cells were fractionated on a DEAE-Sephadex column, and fractions containing RNA polymerase II were pooled and purified further through a phosphocellulose column. Similar to the properties of RNA polymerase II of *Acanthamoeba castellanii* (17), chicken myeloblastosis RNA polymerase II did not adsorb to phosphocellulose matrix and appeared in the flow-through fractions of the column. This procedure removed about 60% of the protein from the polymerase preparations and yet recovered 95% of the enzyme activity. Thus the column purified the enzyme significantly. The phosphocellulose flow-through and the wash fractions containing RNA polymerase activity were combined and subjected to heparin-Sepharose column fractionation (Fig. 1). RNA polymerase II was eluted off the column using a high salt buffer containing 0.8 M ammonium sulfate in TGED. The enzyme fractions were collected and RNA polymerase precipitated by the addition of solid ammonium sulfate. The enzyme was purified further by sedimentation through a 10-40% glycerol gradient (Fig. 2). Based upon the sedimentation values of the markers, the enzyme sedimented at approximately 15.5 S. Table 1

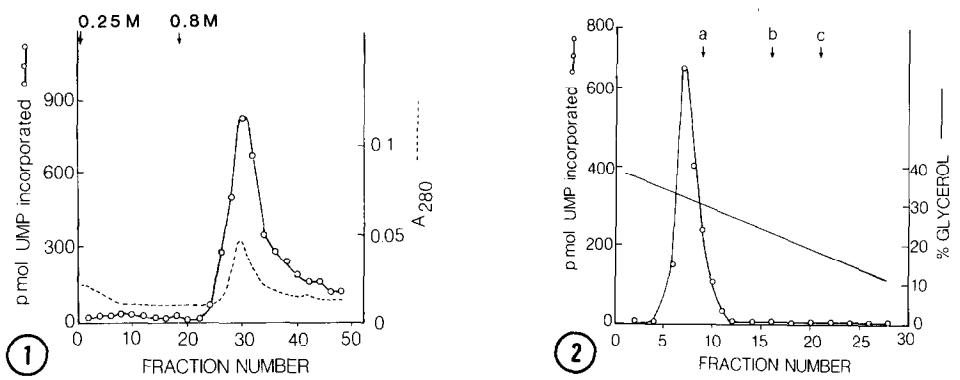

Fig. 1. Heparin-Sepharose chromatography. RNA polymerase activities recovered from the flow-through and wash fractions of phosphocellulose column were combined and further chromatographed on the heparin-Sepharose column as described under Methods. The UMP incorporation represents total pmoles UMP incorporation in 30 min per fraction.

Fig. 2. Glycerol gradient sedimentation. RNA polymerase II was sedimented in 10-40% glycerol gradients as described under Methods. The UMP incorporation represents total pmoles UMP incorporation in 30 min per fraction. a, *E. coli* RNA polymerase (13S); b, chicken myeloblastosis DNA polymerase α (6-8S); c, bovine serum albumin (2-3S).

TABLE 1. Purification of RNA polymerase II from chicken myeloblastosis cells [a]

Fraction	Protein (mg)	Activity (units)	Specific Activity (units/mg)	Specific Activity of Peak Fractions (units/mg)	Yield (%)
Nuclear Extract[b]	23.80	6.64[d]	0.28	(0.28)	-
DEAE-Sephadex[c]	1.20	10.14	8.45	15.75	100
Phosphocellulose[c]	0.48	9.63	20.06	-	95
Heparin-Sepharose[c]	0.087	3.18	36.55	75.10	31
Glycerol Gradient[c]	0.012	1.54	128.33	303.25	15

[a]The data were based on 6.8 g of leukemia cells.

[b]The data presented were total RNA polymerase activity.

[c]The data presented were RNA polymerase II activity.

[d]The total RNA polymerase activity recovered after DEAE-Sephadex chromatography was about 150% of that originally detected in the soluble nuclear extract for the detection of enzyme activity in the nuclear extract was influenced by materials such as nucleases and proteases which were present.

summarizes the overall purification procedure. The enzyme at the peak fraction of the gradient was purified 1083-fold as compared to the crude nuclear extracts, and had a specific activity of 303 units/mg protein. The total yield of purified polymerase II was about 15% of RNA polymerase II eluted off the DEAE-Sephadex column. The purified enzyme appeared as a single homogeneous band as shown by polyacrylamide gel electrophoresis under non-denaturing conditions (data not shown). In addition, the enzyme thus purified was considerably stable and could be stored at -70 °C for several months without a significant loss of enzymatic activity.

Subunit composition of RNA polymerase II from chicken myeloblastosis cells: The subunit structure of RNA polymerase II was determined by electrophoresis of the purified enzyme under denaturing conditions as described under "Methods." The results presented in Figure 3 demonstrate that the purified enzyme contains 7 polypeptide bands with molecular weights (in K-dalton) of 220 (a), 180 (b), 150 (c), 91 (d), 47 (e), 36 (f), and 27 (g), respectively. Subunits c may dissociate into two subunits, c_1 and c_2 of molecular weight 150 KDa and 145 KDa, respectively; the dissociation was more apparent when the SDS-polyacrylamide gel contained urea (data not shown).

Identification of the DNA-binding domain of RNA polymerase II from chicken myeloblastosis cells: The purified RNA polymerase II was concentrated through an Amicon Centricon Microconcentrator (Amicon Corp.), and the concentrated enzyme (12 µg) was subjected to electrophoresis in SDS-polyacrylamide gel (10% acrylamide) containing 4 M urea. The subunits of the enzyme in the gel were then renatured and transferred to nitrocellulose filters by diffusion. After protein transfer, the filters were incubated with ^{32}P-labeled calf thymus DNA and bound radioactivity was located by autoradiography. The chicken nuclear matrix protein and the protein standards

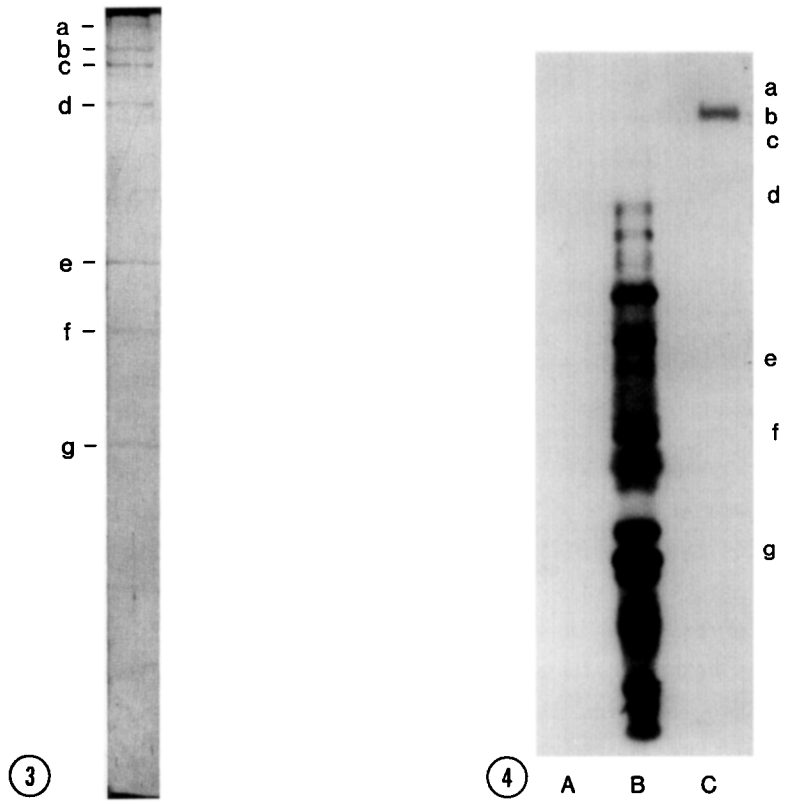

Fig. 3. SDS-polyacrylamide gel electrophoresis of chicken myeloblastosis RNA polymerase II. RNA polymerase II purified from glycerol gradient was concentrated 5-fold using an Amicon Centricon Microconcentrator (Amicon Corp.). About 8-10 µg of the concentrated enzyme was run on a polyacrylamide gel as described under Methods except that the polyacrylamide concentration in the running gel was 12%. The alphabet a through g indicates the positions of RNA polymerase polypeptides of molecular masses (in K-dalton) of 220, 180, 150, 91, 47, 36, and 27, respectively. Densitometer scanning of the gel showed that the molar ratio of 220 KDa:180 KDa was 0.16:1.

Fig. 4. Identification of the DNA-binding polypeptide of RNA polymerase II by the protein blotting method. The procedure was as described under Methods. Lane A, Bio-Rad protein standard (myosin, ß-galactosidase, phosphoylase B, bovine serum albumin and ovalbumin) showing none of the protein bands binds to DNA; lane B, chicken myeloblastosis nuclear matrix proteins (a mixture of DNA-binding proteins) showing positive binding to DNA; lane C, RNA polymerase II showing only one polypeptide band of the enzyme binds to DNA. The alphabet indicates the positions of RNA polymerase II subunits.

(Bio-Rad) were run in parallel lanes of the gel to serve as a positive and a negative control for DNA binding. The results, shown in Figure 4, suggest that only one polypeptide band of chicken myeloblastosis RNA polymerase II binds to DNA while the other subunits show no indication of DNA binding. By filter-staining with Amido Black (16), the polypeptide band was later identified as having a molecular mass of 180 KDa.

Inclusion of protease inhibitors (PMSF, 0.2 mM; pepstatin, 0.7 mg/l; leupeptin, 0.5 mg/l) in the buffer systems during purification significantly

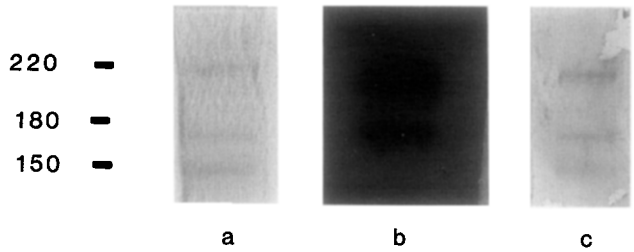

Fig. 5. Binding of 220 KDa and 180 KDa polypeptides of RNA polymerase II to DNA. The procedure was similar to that of Fig. 4C except that the enzyme used was the RNA polymerase II purified in the presence of protease inhibitors (see text). a, Coomassie Blue staining of the large subunits of the gel. The molar ratio of 220 KDa:180 KDa of this preparation was 1:0.42. b, Autoradiogram showing DNA binding subunits 220 KDa and 180 KDa. c, Amido Black staining of the filter after protein transfer.

increased the molar ratio of the 220 KDAa to 180 KDa polypeptide (Fig. 5a), which indicates the possibility that 180 KDa is a proteolytic product of 220 KDa subunit. Incubation of ^{32}P-labeled DNA with RNA polymerase purified in the presence of protease inhibitors and blotted to the filter, however, indicated that both 220 KDa and 180 KDa polypeptides were capable of DNA-binding (Fig. 5b & c). In addition, using a membrane filtration procedure to assay stable DNA-RNA polymerase complex formation (11), it was found that enzymes purified with or without protease inhibitors bind equally well to DNA (data not shown).

DISCUSSION

Extensive purification of RNA polymerase from chicken myeloblastosis cells has been hampered by the large amount of proteolytic enzymes generally present in leukemic cells and by the difficulty in obtaining sufficient amount of starting materials for purification. Whereas large amounts of cells or tissues (kilograms) are employed for the preparation of RNA polymerases from other eukaryotic systems (3), leukemic cells are available in much smaller quantities. In this study, 6 to 8 grams of myelobastosis cells isolated from chicks having leukemia were used for each preparation of RNA polymerase.

The relatively simple and rapid purification procedure reported here enabled us to obtain microgram quantities of RNA polymerase II in a short time to minimize proteolysis. In addition, the protein was of sufficient purity to enable further structural studies. The isolated enzyme had seven polypeptides with molecular weights ranging from 27,000 to 220,000. Of these seven polypeptides, it was not clear whether the 91 KDa was a constituent of the enzyme, and unique to chicken leukemic system, or whether it was a polypeptide copurified with the enzyme. Studies on other eukaryotic systems have shown that polypeptides of 80 KDa - 90 KDa were most commonly found in RNA polymerase III (3).

Carroll and Stollar (18) found that a monoclonal antibody raised against calf RNA polymerase II inhibited enzyme activity by blocking the binding of enzyme to DNA, and was in turn inhibited from binding to the enzyme by DNA. These authors suggested that antibody and DNA binding sites of the enzyme overlap or neighbor each other. The antibody was later found to react specifically with the largest polypeptide of RNA polymerase II, with a preference for the 215,000-dalton subunit over the 180,000-dalton subunit (19). It was then suggested that the subunits protected by DNA from antibody inhibition may be involved in DNA-binding (19,20). However, the direct involvement of a particular RNA polymerase subunit in DNA binding cannot be firmly established by immunological studies for it is not known whether DNA blocks the important antigenic site in these experiments or changes the enzyme conformation (20). Direct DNA-enzyme binding studies have been reported by Horikoshi et al. (21), which showed that the largest subunit of RNA polymerase II from Ehrlich Ascites cells bound DNA. The largest subunit in this case, however, was 165 KDa, much smaller in size than the two largest subunits commonly found in other eukaryotic systems. Using a similar DNA-polypeptide binding procedure we found that both 220 KDa and 180 KDa polypeptides, but not any other subunits, of RNA polymerase II bound to DNA. The presence of only one DNA-binding band (180 KDa) in Fig. 4 may reflect that proteolysis of the largest subunit during purification does occur in this system, and that the limited amount of 220 KDa in the preparation did not permit sufficient protein transfer for subsequent DNA-binding to occur. Therefore, these studies suggest that certain structural and functional domains necessary for DNA-binding for RNA polymerase II must reside in the 180,000-dalton polypeptide of the subunit and that the C-terminal sequence of RNA polymerase II largest subunit (9) may not be an essential factor contributing to DNA binding.

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