Molecular Structures of DNA-Dependent RNA Polymerases (II) from Calf Thymus and Rat Liver

(acrylamide gel electrophoresis/sodium dodecyl sulfate/subunits/polymerase II)

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ABSTRACT DNA-dependent RNA polymerase II has been purified to high specific activity and apparent homogeneity from both calf thymus and rat liver. Two form II enzymes are present in rat-liver preparations, one with the molecular structure [(190,000)(150,000)(35,000)(25,000)], the other with a molecular structure of [(170,000)(150,000)(35,000)(25,000)]. Molecular weights are within ±5% but the absolute values are approximate. Inclusion of a proteolytic inhibitor during the isolation procedure decreases the proportion of the molecule containing the 170,000 subunit. Calf-thymus RNA polymerase preparations typically exhibit four components on polyacrylamide gels that contain sodium dodecyl sulfate, with an apparent molecular structure of [(190,000)(150,000)(35,000)(25,000)]. In addition, some calf-thymus polymerase II preparations contain small quantities of the [(170,000)(150,000)(35,000)(25,000)] species; the quantity of this species may also be increased from less than 5% in the normal preparation to at least 40% in an “aged” preparation. Thus, the 170,000 subunit may be derived from the 190,000 subunit in both tissues. Until unequivocal evidence is obtained on this point, however, the possibility that the large subunits are unique species should not be eliminated. The general structural similarity of the eukaryotic RNA polymerase II with that of the prokaryotic polymerase suggests that the modes of action and regulation may be analogous.

Multiple forms of DNA-dependent RNA polymerases have been shown to exist in eukaryotic cells (1, 2). Polymerase I is localized in the nucleolus (2); its RNA product has the base composition and hybridization behavior expected of ribosomal RNA (3). These data indicate that the major role of this enzyme is to synthesize ribosomal RNA, although it may transcribe a limited number of other RNA species. Polymerase II, on the other hand, is found in the nucleoplasm (2). Its product has a more DNA-like base composition and is competed well in hybridization-competition experiments by whole nuclear RNA (3), suggesting that this polymerase synthesizes the bulk of the nucleoplasmic RNA species. A role for polymerase III, believed to be present in the nucleoplasm, has yet to be elucidated. The mushroom toxin, α-amaminin, selectively inhibits polymerase II, while forms I and III are not affected (4, 5). The general properties of these enzymes suggest that they are distinct molecules, but a common component(s) is not ruled out.

Blatti et al. (3) reported the purification of calf-thymus polymerase II; preliminary structural analysis suggested that the molecule contained at least three different subunits. In this paper, we report the isolation of polymerase II from calf thymus, as well as from rat liver. The present studies suggest that the enzymes have similar structures and contain four, not three, components. Furthermore, two species of polymerase II, differing at least in the large molecular weight component, are present in the preparations from both calf thymus and rat liver. Preliminary experiments suggest that one of the forms may be derived from the other.

METHODS

Purification procedure

Solutions. Buffer A: 1.0 M sucrose–10 mM Tris (pH 7.9)–25 mM KCl–3 mM CaCl2–5 mM MgCl2–1 mM spermine.

TMSD buffer: 0.01 M Tris (pH 7.9)–0.05 M MgCl2–1.0 mM sucrose–1 mM dithiothreitol (S2 threitol).

TGMED: 0.05 M Tris (pH 7.9)–25% glycerol–5 mM MgCl2–0.1 mM EDTA–0.5 mM S2 threitol.

TKMS: 0.01 M Tris (pH 7.9)–25 mM KCl–5 mM MgCl2–1 mM spermine.

Preparation of Nuclei. The appropriate amount of 4 M (NH4)2SO4 (pH 7.9) (measured on an aliquot diluted 1:10) was added to the indicated buffers.

A. Calf thymus: The thymus glands from 2 to 8-week-old calves were removed immediately after slaughter, placed in ice, and transferred to the laboratory. 1 kg of thymus was mixed with 1 liter of Buffer A and homogenized for 15 sec in a large Waring blender at a low setting. 350 ml of Buffer A was added to every 500 ml of homogenate and the mixture was further homogenized in a blender (Osterizer) for 3 min at a low setting. This suspension was passed through one layer of cheese cloth to remove connective tissue, the homogenate was underlain with about 1/6 volume of 2.0 M sucrose (containing Buffer A), and the nuclei were then sedimented by centrifugation at about 13,000 × g (Sorvall RC-2B, GS head, 9500 rpm) for 30 min. The nuclei were then suspended in a volume of TMSD buffer equal to the volume of the original tissue. This nuclear suspension can be either stored frozen or used directly in the next steps.

B. Rat liver: Rat liver nuclei were prepared by a modification of the method of Roeder and Rutter (1). 400 g of rat

Abbreviation: S2 threitol, dithiothreitol.

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DEAE-Sephadex Chromatography. The solubilized enzyme solution was loaded onto an A-25 DEAE-Sephadex column (5 mg of protein per ml of bed volume) that had been washed with TGMED containing 0.03 M \((\text{NH}_4)_2\text{SO}_4\). The column was then eluted with 2 bed-volumes of a linear gradient of 0.03–0.6 M \((\text{NH}_4)_2\text{SO}_4\) in TGED containing 0.2 mg/ml of bovine serum albumin. About 40 fractions were collected; each tube had enough MgCl\(_2\) to bring the final concentration to 5 mM Mg\(^{2+}\). Polymerase II activity was eluted in about fractions 17–26, the peak corresponding to 0.12 M \((\text{NH}_4)_2\text{SO}_4\).

DNA–Sepharose Chromatography. The polymerase II from 400 g of rat liver or 1 kg of calf thymus, purified through the phosphocellulose step, was dialyzed about 8 hr against TGMED containing 0.01 M ammonium sulfate. This solution was then loaded on a 25-ml column of DNA–Sepharose, prepared by the method of Poonian et al. (6) from denatured calf-thymus DNA and Sepharose activated by cyanogen bromide. The column was eluted with 125 ml of a 0.01–0.3 M linear gradient of ammonium sulfate in TGMED. 40 fractions were collected; the activity was eluted in fractions 13–22, corresponding to about 0.10 M \((\text{NH}_4)_2\text{SO}_4\).

Sucrose-gradient Centrifugation. The combined fractions from the Sepharose step were dialyzed against TGMED, to an ionic strength equivalent to 0.03 M \((\text{NH}_4)_2\text{SO}_4\), then placed on a DEAE-Sephadex column (10 mg of protein per ml of bed volume) pre-equilibrated with TGMED. The enzyme was eluted in a concentrated solution by stripping the column with TGMED containing 0.8 M \((\text{NH}_4)_2\text{SO}_4\). The solution was then dialyzed against TGMED containing 0.3 M \((\text{NH}_4)_2\text{SO}_4\) for the calf-thymus enzyme or 0.15 M \((\text{NH}_4)_2\text{SO}_4\) for the rat-liver enzyme.

A 0.5-ml aliquot was applied to 5–20% sucrose gradient in TGMED containing 0.3 M \((\text{NH}_4)_2\text{SO}_4\) (rat liver, 0.15 M) and centrifuged for 50 hr at 40,000 rpm and 4°C in a Spinco SW40 rotor. After centrifugation, the contents of each tube were collected in about 25 fractions and assayed. Sedimentation coefficients were estimated by comparison with sedimentation of pyruvate kinase, 10.0 S (7); yeast phosphofructokinase, 17.9 S (8) and Escherichia coli RNA polymerase, (holoenzyme) (12).

RESULTS AND DISCUSSION

Purification of polymerases from calf thymus and rat liver

The purification procedure detailed in the Methods section is summarized in Table 1. The procedure through the DEAE-Sephadex step is similar to that developed by Roeder and Rutter (1). The additional purification steps involve phosphocellulose and DNA–Sepharose chromatography, and finally sucrose gradient sedimentation.

Polymerase II preparations from both calf thymus and rat liver exhibit relatively high specific activities: various preparations have ranged from 400–1000. This is similar to the specific activity of the E. coli RNA polymerase plus sigma factor with this template (9). Using the specific activity of the purified enzyme, and an assumed molecular weight of 400,000, we can estimate from the activity in the initial extracts that the concentration of polymerase II is 800 molecules per calf-thymus nucleus, and 10,000 molecules per rat-liver nucleus. From the data of Keller and Goor (11) we calculate there are about 50,000 molecules of polymerase II per KB cell-nucleus. These values can be compared to an estimated 3000 RNA polymerase molecules per E. coli cell. Since mammalian cells contain about 1000 times as much DNA as prokaryotic cells, it is obvious that the concentra-
TABLE 1. Preparation of polymerase II from calf thymus and rat liver

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total units (nmol UMP incorporated/10 min)</th>
<th>Specific activity (units/mg protein)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thymus</td>
<td>Liver</td>
<td>Thymus</td>
</tr>
<tr>
<td>1. Nuclear homogenate</td>
<td>150</td>
<td>540</td>
<td>0.05</td>
</tr>
<tr>
<td>2. Enzyme solubilization</td>
<td>35</td>
<td>540</td>
<td>0.17</td>
</tr>
<tr>
<td>3. DEAE-Sephadex chromatography</td>
<td>60</td>
<td>540</td>
<td>10</td>
</tr>
<tr>
<td>4. Phosphocellulose chromatography</td>
<td>20</td>
<td>270</td>
<td>200</td>
</tr>
<tr>
<td>5. DNA–Sepharose chromatography</td>
<td>17</td>
<td>160</td>
<td>500</td>
</tr>
<tr>
<td>6. Sucrose-gradient sedimentation</td>
<td>17</td>
<td>160</td>
<td>500*</td>
</tr>
</tbody>
</table>

The assay of enzyme activity was described (3). The assay mixture contained [3H]UTP (New England Nuclear Corp.); 0.83 Ci/mmole. Protein concentration of all solutions except the sucrose-gradient fractions was determined by the method of Lowry et al. (10). The protein concentration of the sucrose-gradient fractions was determined by summing the intensities of all bands stained with Coomassie blue on sodium dodecyl sulfate–polyacrylamide gels and comparison with a standard curve of the intensities of known quantities of bovine serum albumin electrophoresed in parallel gels. One unit of activity is defined as 1 nmol incorporated per 10 min; the specific activity is expressed as units/mg of protein.

*Average of 20 preparations, range from 400 to 600 (see text).
† Average of 8 preparations, range from 400 to 1000 (see text).

Electrophoresis of the native enzyme at high pH

The calf-thymus and rat-liver polymerase II preparations have been electrophoresed on 5% polyacrylamide gels at pH 8.5. These conditions presumably maintain the molecule in its native state and, hence, are termed “native” gels. Figs. 1a and 1b show the patterns for the two enzyme preparations. A single band was observed with the calf-thymus enzyme; major and minor band were evident in the gels run with the rat-liver polymerase. The two bands obtained from rat-liver polymerase were eluted from the gels by finely mincing the stained gel slices in sodium dodecyl sulfate solutions. These species were then rerun on sodium dodecyl sulfate gels as described below, and showed identical band patterns. It is concluded that the two molecular species are very similar, if not identical. Thus, the two bands may represent different states of aggregation or, alternatively, differently charged derivatives of the enzyme.

Molecular weight of the enzymes

Sedimentation of the polymerases with marker proteins [pyruvate kinase, 10 S; fructose-6-phosphate kinase, 17.9 S; and E. coli RNA polymerase, (holoenzyme) 15.0 S] indicates a sedimentation coefficient of 14.5–15 S for calf-thymus and rat-liver enzymes. The sedimentation properties of these enzymes are very similar to that of prokaryotic polymerase. Minimum molecular weight values calculated from these s values, on the assumption of a globular shape, are about 350,000–400,000. The molecular weight of the E. coli core polymerase is about 380,000 (12)–400,000 (13).

![Fig. 1. Gel electrophoresis of polymerase II from calf thymus and rat liver. 5% acrylamide gels (pH 9) were prepared by the method of Shapiro et al. (14). 0.10-ml samples were dialyzed against 50 mM Tris-phosphate (pH 8.9)–10% glycerol–1.0 mM S,S-threitol containing 0.10 M (NH₄)₂SO₄ for the calf-thymus enzyme (1a) and 0.05 M (NH₄)₂SO₄ for the rat-liver enzyme (1b). These were layered onto the gels and electrophoresed at 3 mA/gel.](image-url)
Molecular subunit structures of the enzymes

The polymerase preparations were electrophoresed on sodium dodecyl sulfate gels. This method dissociates complex proteins into their polypeptide chains, and has been successfully used in the determination of the subunits of several enzymes, including the E. coli polymerase (12, 13). The precision of the measurement of component molecular weight is (±5%), but the absolute accuracy, especially for molecules over 100,000, is questionable, due to the lack of availability of appropriate standards. Even the molecular weight values for the E. coli enzyme subunits used as standards are subject to some uncertainty (12, 13).

After sedimentation on sucrose density gradients, fractions from the peak of polymerase activity were electrophoresed. Calf-thymus RNA polymerase preparations exhibit four major components in sodium dodecyl sulfate gels; the amount of each component parallels the polymerase activity profile (Fig. 2). A similar enzyme preparation was dissociated in 6 M guanidine- HCl, dialyzed against 6 M urea, and then dialyzed against 1% sodium dodecyl sulfate; the gel pattern obtained was identical to that presented here, both with respect to molar ratios and molecular weights of the different components. These experimental results suggest that these components are the ultimate dissociation products of the enzyme. The molecular weights of the bands were estimated to be 190,000, 150,000, 35,000, and 25,000 by comparison of their mobilities to those of marker proteins (bovine serum albumin and E. coli/RNA polymerase subunits) according to the procedure first described by Shapiro et al. (14). Densitometer tracings of the gels stained with Coomassie blue suggested equivalent molar ratios of each component. The minimum molecular weight estimated from the sedimentation coefficient (370,000) compares favorably with the molecular weight of a structure containing 1 mol of each of the components observed on dissociating gels: [(190,000), (150,000), (35,000), (25,000) = 400,000]. From these collected observations, we conclude that the four components observed are subunits of calf-thymus polymerase II. In most calf-thymus preparations, a component of about 170,000 molecular weight is present in relatively small proportions. However, in preparations allowed to age at 4°C in the crude state, sodium dodecyl sulfate gel analysis of the final product revealed five components [(190,000)0.6 (170,000)0.4 (150,000)0.1 (35,000)0.1 (25,000)]. This result suggests the presence of two enzymatic species, one possibly formed from the other during the long incubation period.

The rat-liver polymerase showed a more complex gel pattern (Fig. 3): five components were observed coincident with the peak of activity. The molecular weights are (±5%) (190,000)x, (170,000)x, (150,000)y, (35,000)z, (25,000). Thus, four of the subunits are similar, but not necessarily identical, to those of the calf-thymus enzyme. The molar ratios of the three lower molecular weight components are equivalent; the molar equivalents of the higher molecular weight components (190,000 and 170,000) always sum to one. The relative amount of the 190,000 component varies from preparation to preparation, from 0.05–0.6, with the remainder of the integral value being made up of the 170,000 component.

In other gels or within the same gels as the eukaryotic polymerases. (b) Densitometer tracing of the gel (tube 9) represented in Fig. 2a.
There is no apparent change in the specific activity of the final preparations with changes in the molar ratio of the 190,000 and 170,000 components. As reported above, the sedimentation coefficient of the rat-liver polymerase is similar to that of the calf-thymus enzyme, and not very different from the E. coli enzyme. This sedimentation coefficient, together with the molar ratios of the subunits, precludes the presence of all five components in one structure. In contrast, the combined data suggest the presence of two enzymes: one [(190,000), (150,000), (35,000), (25,000)] similar to the calf-thymus enzyme, and the other [(170,000), (150,000), (35,000), (25,000)]. These two enzymes would have molecular weights of about 400,000 and 380,000, and could have similar sedimentation coefficients to produce the observed coincident subunit patterns after sucrose density gradient centrifugation. A change in the proportion of the two enzymes in the various preparations explains the observed variation in the amount of the 170,000 and 190,000 molecular weight components in these preparations.

We conclude that the five components observed are subunits of two similar polymerases. Kedinger et al. (15) as well as Mandel and Chambon (16) and Chesterton and Butterworth (17) have recently presented preliminary evidence supporting a similar conclusion. They have partially resolved polymerases with different high molecular weight "subunits" (200,000–215,000 and 150,000–160,000) and (180,000–185,000 and 150,000–160,000), respectively; their work did not include an analysis of the small molecular weight components. Although there is a discrepancy in the estimates of the molecular weight of the subunits, it seems clear that these workers are dealing with the same polymerase species whose structure is described in the present paper. Whether both enzymes exist in rat-liver nuclei, or whether one is produced by degradation of the 190,000 subunit to the 170,000 subunit during isolation is not yet clear. However, in preparations in which phenylmethylsulfonyl fluoride was included to inhibit proteolysis, the molar ratios of the 190,000 component varied from 0.4–0.6 in three preparations, whereas the average value was 0.1 for those in which the inhibitor was absent. Such a modification need not preclude a specific function for the two enzymes. Leighton et al. have recently shown § that a specific proteolytic modification of RNA polymerase occurs during sporulation of Bacillus subtilis.

Thus, proteolysis is a possible mechanism for creating two forms of polymerase II. However, the putative modification must be very specific, yielding only one product (the 170,000 subunit) from the 190,000 subunit. Furthermore, it conserves activity, since high specific activity enzyme has been obtained that contains very little 190,000 subunit. Although our data are consistent with proteolytic conversion, they must be considered tentative, and do not rule out the possibility that the two large subunits may be products of separate genes. Because of the uncertainty of the origin and function of these multiple forms of polymerase II, we believe it is premature to propose a specific nomenclature (such as Ia and Ib).

The small degree of heterogeneity detected in polymerase II, coupled with the fact that this enzyme transcribes a large proportion of the nuclear RNA, indicates that much of the specificity for gene transcription resides elsewhere.

The present studies, however, do indicate a general structural similarity between the isolated eukaryotic RNA polymerase II and the prokaryotic "core" polymerase. This similarity suggests that the general regulatory rules for RNA polymerases in prokaryotes and eukaryotes may also be similar. If indeed the isolated polymerase II is an analogue of the core polymerase, then there may also be the eukaryotic analogue of the σ factor.

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