Purification and lipid-layer crystallization of yeast RNA polymerase II

(temperature-sensitive mutant/*in vitro* transcription/monoclonal antibody/immunoaffinity chromatography/electron crystallography)

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ABSTRACT Yeast RNA polymerase II was purified to homogeneity by a rapid procedure involving immunoaffinity chromatography. The purified enzyme contained 10 subunits, as reported for conventional preparations, but with no detectable proteolysis of the largest subunit. In assays of initiation of transcription at the yeast *CYC1* promoter, the enzyme complemented the deficiency of an extract from a strain that produces a temperature-sensitive polymerase II. Mammalian RNA polymerase II was inactive in this initiation assay. The purified yeast enzyme formed two-dimensional crystals on positively charged lipid layers, as previously found for *Escherichia coli* RNA polymerase holoenzyme. Image analysis of electron micrographs of crystals in negative stain, which diffracted to about 30-Å resolution, showed protein densities of dimensions consistent with those of single polymerase molecules.

RNA polymerase II (pol II) is responsible for the synthesis of mRNA precursors in eukaryotes. The most purified preparations of the yeast and mammalian enzymes contain 10 polypeptides, ranging in size from 10 to 220 kDa (1, 2). At least four further components are required for accurate initiation at pol II promoters. By contrast, *Escherichia coli* RNA polymerase holoenzyme is made up of only four distinct polypeptides but is alone capable of recognizing a promoter and initiating transcription (3). One of the *E. coli* polypeptides, the σ subunit, is primarily responsible for promoter selection, while other subunits are involved in DNA and RNA binding, catalysis, and regulation. Which polypeptides play corresponding roles in pol II? Which subunit or accessory factor serves as the counterpart of the σ subunit of the *E. coli* enzyme? What are the functions of the other factors? Such questions call for the isolation of fully functional pol II and the elucidation of its structure and mechanism of action.

The yeast *Saccharomyces cerevisiae* is advantageous for studies of pol II transcription. Genes encoding all but one of the subunits have been isolated (R. A. Young, personal communication), and altered forms have been obtained by selection and by *in vitro* mutagenesis (4, 5). Many yeast pol II promoters have been analyzed in detail, and genes and proteins involved in both positive and negative regulation have been identified. A yeast nuclear extract is readily prepared that will support accurate initiation of transcription from a number of pol II promoters and that exhibits some aspects of regulation (6–8). The abundant source of material should allow the isolation of pol II and accessory factors in amounts sufficient for structural and mechanistic studies.

Many aspects of pol II transcription are conserved across species, so results from the yeast system should be applicable

to the process in higher eukaryotes. The two largest subunits of yeast pol II are similar in amino acid sequence to those of the *E. coli*, *Drosophila*, and murine enzymes (9–12). Pol II is distinctive in possessing a repeated heptapeptide at the C terminus of the largest subunit, and the sequence of this heptapeptide is identical in the yeast and mammalian enzymes (13). Yeast TATA-binding protein will substitute for the corresponding factor from HeLa cells in the initiation of transcription *in vitro* (14, 15), and gene-activator proteins are functionally interchangeable between yeast and human cells and extracts (16, 17). A mammalian pol II promoter is transcribed with comparable efficiency in yeast and HeLa extracts (18).

The published method of purifying yeast pol II involves three chromatographic steps and a glycerol gradient, giving enzyme free of contaminants in 20–30% yield. The product is heterogeneous, due to partial proteolysis of the largest subunit, which in the case of the mammalian enzyme results in forms lacking part or all of the C-terminal repeat region. The purified yeast enzyme is capable of nonspecific initiation and chain elongation but has not been tested for accurate initiation at promoters for lack of a suitable assay. We report here a rapid method of purifying yeast pol II with an intact C-terminal domain, based on a recent innovation in immunoaffinity chromatography (N.E.T., D. B. Aronson, and R.R.B., unpublished work). We describe an assay for specific initiation by pol II that employs an unfractionated nuclear extract from a pol II mutant strain. Finally, we show that purified enzyme active in this assay forms two-dimensional crystals.

MATERIALS AND METHODS

Yeast Strains. Pol II was prepared from *S. cerevisiae* strain BJ926 (α/a *trp1*+/+ *prc1*-126/*prc1*-126 *pep4*-3/*pep4*-3 *prb1*-1122/*prb1*-1122 *can1*/*can1*), kindly provided by E. Jones (Carnegie-Mellon University, Pittsburgh). Nuclear extract containing temperature-sensitive pol II (pol II^{ts}) was prepared from strain Y260-1 (*trp1* *leu2*-3,112 *his4*-580 *ura3*-52, *pep4*-3 *rpb1*-1), a derivative of strain Y260, which was kindly provided by R. Young (Massachusetts Institute of Technology, Cambridge, MA).

Purification of Yeast Pol II. Yeast cells were grown in YPD medium (1% yeast extract/2% Bacto-peptone/2% glucose) to an OD₆₆₀ value of 8 (measured at 1:200 dilution in a Hewlett-Packard 8451A spectrophotometer) and harvested by centrifugation in a Beckman JA-10 rotor at 5000 rpm for 5 min at 4°C. The cells were washed once in cold water, suspended in 0.2 vol (vol/wet weight of cells; about 165 g of cells from 20 liters of culture) of 0.25 M TrisCl, pH 7.9/5 mM EDTA/2.5 mM dithiothreitol/10 mM sodium pyrophosphate/5% dimeth-

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Abbreviation: pol II, RNA polymerase II.

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yl sulfoxide/50% (vol/vol) glycerol/protease inhibitors,[§] and frozen in liquid nitrogen. The frozen cell suspension (200 ml) was thawed, disrupted with 150 ml of glass beads by fifteen 30-sec bursts of a bead beater (Biospec Products, Bartlesville, OK) with 90 sec of cooling between bursts, and diluted to 350 ml with buffer A (50 mM TrisCl, pH 7.9/1 mM EDTA/0.5 mM dithiothreitol/10 mM NaF/10 mM sodium pyrophosphate/10% glycerol/protease inhibitors[§]). Cell debris was removed by centrifugation in a Sorvall SS-34 rotor at 17,000 rpm for 30 min at 4°C. The supernatant was filtered through Whatman 3MM paper and applied to a heparin-Sepharose CL-6B column (5 × 7.5 cm; Pharmacia) in buffer A. The column was washed with 0.1 M KCl in buffer A until the protein concentration in the effluent [determined by the method of Bradford (19)] was <0.05 mg/ml (about 300 ml of wash buffer). Bound material was eluted with 0.6 M KCl in buffer A until the protein concentration was <0.1 mg/ml (about 150 ml). Protein in the eluate was concentrated by the addition of solid ammonium sulfate to 50% of saturation, followed by centrifugation in a Sorvall SS-34 rotor at 17,000 rpm for 30 min at 4°C. The pellet was dissolved in buffer B (50 mM TrisCl, pH 7.5/1 mM EDTA/10 mM NaF/protease inhibitors[§]) to give a conductivity corresponding to an ammonium sulfate concentration of 0.5 M (about 15 ml) and passed through a DEAE-Sepharose column (2.5 × 7 cm; Pharmacia) in buffer B containing 0.5 M ammonium sulfate, to remove nucleic acids. The solution was adjusted to an ammonium sulfate concentration of 0.2 M with buffer B, combined in a 50-ml screw-cap tube with 3 ml of monoclonal antibody 8WG16-Sepharose (20), and turned gently end-over-end for 1 hr at 4°C, with care to avoid bubble formation. The 8WG16-Sepharose was collected by centrifugation at 4000 × g for 5 min at 4°C and washed five times with 15-ml portions of 0.2 M ammonium sulfate in buffer B for a total of 30 min at 4°C. Bound material was then eluted from the 8WG16-Sepharose in 3 ml of 30% glycerol/0.5 M ammonium sulfate in buffer B for 15 min at room temperature. This step was repeated three times and the eluates were pooled (eluate 1). Additional material was removed from the 8WG16-Sepharose by two elutions with 70% glycerol in buffer B (eluate 2). Eluates 1 and 2 contained 56% and 26% of the protein that bound to the antibody-Sepharose, respectively. Eluate 2 appeared slightly more enriched for pol II by SDS/PAGE and was further processed to remove minor contaminants. This eluate was dialyzed against buffer C (50 mM TrisCl, pH 7.5/1 mM EDTA/0.5 mM dithiothreitol/10 mM NaF/20% glycerol/protease inhibitors[§]) to a conductivity corresponding to 0.1 M ammonium sulfate and then fractionated by HPLC on a Bio-Gel SEC DEAE-5 PW column (7.5 × 75 mm; Bio-Rad) equilibrated with 0.1 M ammonium sulfate in buffer C. After elution with 0.3 M ammonium sulfate in buffer C, fractions containing pol II activity were pooled and dialyzed against 50 mM TrisCl, pH 7.5/1 mM EDTA/0.5 mM dithiothreitol/50% glycerol until the conductivity was <20 mS/cm. The purified pol II was stored in liquid nitrogen and could be thawed once with no loss of activity in the initiation assay.

Assay for Accurate Initiation by Pol II. Extract was prepared from strain Y260-1 as described (6, 21) for strain BJ926 ("wild type"), except that cells and spheroplasts were maintained at 24°C rather than 30°C. Transcription was performed as described (18) in 30- μ l reaction mixtures containing 4 μ l of nuclear extract (15–20 mg of protein per ml of nuclear extract). The template was pGAL4CG– (18), with a GAL4

protein-binding site upstream of the yeast *CYC1* promoter fused to a 377-base-pair sequence lacking guanosine residues on the coding strand. Transcripts of the G-minus sequence were isolated and analyzed as described (18) and were quantified with an Ambis radioanalytic imaging system (Ambis Systems, San Diego).

Two-Dimensional Crystals of Pol II. Two-dimensional crystals were formed from pol II (150 μ g/ml) in 10 mM TrisCl, pH 7.5/50 mM ammonium sulfate/0.2 mM EDTA/2 mM spermidine/8% (vol/vol) glycerol, transferred to electron microscope grids, and analyzed by electron microscopy and image analysis as described (22–25).

For electron microscopy of single molecules, pol II was diluted to 10 μ g/ml in the buffer used for crystallization and incubated for a few minutes on carbon-coated, glow-discharged electron microscope grids, washed with one drop of 10 mM TrisCl (pH 7.5), and stained with 1% (wt/vol) uranyl acetate. Micrographs were recorded under minimal dose conditions at a magnification of ×46,000.

RESULTS

Purification of Yeast Pol II. An extract was prepared from a protease-deficient strain of yeast by homogenization with glass beads. Most of the purification of pol II from this extract was achieved in two steps, elution with 0.6 M KCl from heparin-Sepharose and chromatography on a monoclonal antibody column. The preparation was concentrated between these steps by precipitation with ammonium sulfate, and a few remaining contaminants were finally removed by HPLC on a DEAE-5 PW column. Starting from 20 liters of cell culture, nearly a milligram of immunoaffinity-purified pol II was obtained in a day in 60% yield (Table 1).

The particular monoclonal antibody column used has two especially useful properties (N.E.T., D. B. Aronson, and R.R.B., unpublished work). First, the antibody is directed against the C-terminal repeat region of the largest subunit, resulting in the enrichment of pol II that contains this region in an unproteolyzed form. Second, pol II binds tightly to the column in 0.2 M ammonium sulfate at 4°C but is released upon the addition of glycerol (30–70%) and raising the ammonium sulfate concentration (to 0.5 M) and temperature (to 23°C). The enzyme can therefore be eluted from the antibody column with protein-stabilizing agents, rather than with denaturants.

Nonspecific initiation/chain-elongation activity was measured by transcription of poly(G) from a poly(C) template (26). Immunoaffinity/HPLC-purified pol II incorporated 390 nmol of nucleotide per mg of protein per min at 30°C. A specific activity of 400 nmol of nucleotide per mg per min has been reported for the enzyme prepared by conventional methods (27, 28).

The fraction of pol II molecules in our preparations capable of nonspecific initiation/chain elongation was determined by the method of Kadesch and Chamberlin (29). Pol II was allowed to initiate on an oligo(dC) tail at the 3' end of a duplex DNA molecule. With an excess of tailed duplex over pol II

Table 1. Purification of yeast pol II

Step	Vol, ml	Protein, mg	Total units*	Specific activity, units/mg	Yield, %
Crude extract	250	5000	259	0.052	100
Heparin-Sepharose	150	150	268	1.79	100
Ammonium sulfate	10	75	232	3.09	86
Immunoaffinity	1.8	0.72	161	224	60

*Nonspecific initiation/chain-elongation activity was assayed as described (26). A unit of activity is defined as 1 nmol of nucleotide incorporated per min.

[§]Per liter, 10 mg of aprotinin, 320 mg of benzamidin, 1 mg of pepstatin, 10 mg of leupeptin, 5 mg of L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone, 24 mg of L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride, and 174 mg of phenylmethanesulfonyl fluoride.

molecules, and with the addition of heparin (0.1 mg/ml) to prevent reinitiation, the number of transcripts obtained corresponds to the number of active pol II molecules. Immunoaffinity/HPLC-purified pol II contained 42% active molecules (average of three determinations), which compares favorably with a value of 18% for purified calf thymus pol II (gift of C. Kane, University of California, Berkeley, CA) tested alongside. Published values for the calf thymus enzyme are from 15% to 25% active molecules (29).

SDS/PAGE of the immunoaffinity/HPLC-purified enzyme (Fig. 1) revealed 10 polypeptides of 205, 150, 45, 34, 30, 26, 21, 18, 16, and 12.5 kDa. There was no detectable degradation of the largest polypeptide. A shoulder on the leading edge of the second-to-fastest migrating band may be indicative of a degree of heterogeneity in this subunit, as has been reported (31).

Assay for Accurate Transcription Initiation by Pol II *in Vitro*. Initiation by purified pol II was assayed by complementing the deficiency of a nuclear extract from a mutant strain. This strain is temperature-sensitive due to a mutation in the gene for the largest subunit of pol II (21). A whole-cell extract from this strain lacks pol II activity in nonspecific initiation/chain elongation (21). We found, however, that a nuclear extract possessed significant activity in specific initiation at the *CYC1* promoter. This activity was temperature-sensitive, in contrast with the activity of an extract from a wild-type strain (Fig. 2 *Top and Middle*).

Addition of immunoaffinity/HPLC-purified pol II to heat-treated extract from the temperature-sensitive mutant strain restored the capacity to initiate transcription at the *CYC1* promoter (Fig. 2 *Top*). The amount of transcription was linearly dependent on the amount of purified pol II added (Fig. 2 *Bottom*). Other pol II preparations were also assayed in this way. When amounts of yeast pol II prepared by conventional methods (27) and by the present procedure having equal activities in nonspecific initiation/chain elongation were compared, the conventional preparation was approximately half as active in the specific initiation assay. Neither calf thymus pol II [gift of C. Kane, purified by the method of Hodo and Blatti (2)] nor partially purified rat pol II (gift of R. Conaway and J. Conaway, Oklahoma Medical Research Foundation, Oklahoma City) showed detectable activity in the yeast initiation assay, although both enzymes are capable of specific initiation in mammalian transcription systems (data not shown).

Two-Dimensional Crystals of Pol II. *E. coli* RNA polymerase holoenzyme forms two-dimensional crystals on positively charged lipid layers (22). We have found that immunoaffinity/HPLC-purified yeast pol II can be crystallized in

FIG. 1. Subunit composition of immunoaffinity/HPLC-purified yeast pol II. The enzyme (10 μ g) was electrophoresed in a SDS/15% polyacrylamide gel and stained with Coomassie brilliant blue as described (30). The gel (Lower, direction of electrophoresis from right to left) was scanned at 633 nm with an LKB Ultrosan XL laser densitometer. The absorbance scale on the left (right) is for the scan left (right) of the hash marks.

FIG. 2. Assay for initiation of transcription by complementation of a pol II-deficient extract. (*Top*) Heat-inactivation of transcriptional activity of a pol II^{ts} extract, and restoration of activity by the addition of purified pol II. Transcription was performed with unheated (lane 1) or heat-treated (42°C for 18 min; lanes 2–4) nuclear extract from strain Y260-1. Immunoaffinity/HPLC-purified pol II (7 and 35 ng) was added as indicated (lanes 3 and 4) to the heat-treated extract prior to transcription. Bands due to accurately initiated transcripts are indicated by the bracket. (*Middle*) Time course of heat-inactivation of transcriptional activity of pol II^{ts} (◆) and wild-type (□) extracts. Levels of transcription are expressed as percentages of the value for an unheated extract. Error bars indicate the range of data from three separate experiments. (*Bottom*) Dependence of transcriptional activity of heat-treated pol II^{ts} extract upon amount of immunoaffinity/HPLC-purified pol II added.

the same way (Fig. 3). Electron micrographs of crystalline areas in negative stain gave diffraction extending to about 30-Å resolution (Fig. 4).

Images of four crystalline areas, containing from 100 to 300 unit cells, were processed and averaged. Indexed as shown in Fig. 4, the average unit-cell parameters were $a = 529 \pm 7$ Å, $b = 232 \pm 2$ Å, and $\gamma = 92 \pm 2^\circ$. Projection maps of the noise-filtered images, calculated without the imposition of symmetry, were strongly suggestive of C222 symmetry, consistent with the absence of reflections for odd ($h + k$), the

FIG. 3. Electron micrograph of an ordered array of yeast pol II on an octadecylamine/egg phosphatidylcholine layer, stained with uranyl acetate. The edge of a lipid patch is shown running diagonally from left to top. Single, disordered pol II molecules adsorbed to the carbon surface of the electron microscope grid are seen in the upper left corner, demonstrating that ordered areas formed only on lipid patches. The width of the micrograph corresponds to 670 nm.

apparent *mm* symmetry of the diffraction pattern, and the included angle of 90° . On refinement of the origin of the reciprocal lattice by assuming *P2* symmetry, an average phase error (based on comparison of individual phases with the nearest centrosymmetric value) of 14° was obtained when reflections with amplitudes greater than twice background were included to a resolution of 30 Å. Imposing *C222* symmetry resulted in an average phase error of 15.8° and an

FIG. 4. Computed diffraction pattern, corrected for lattice distortions, of the crystalline area shown in Fig. 3. The reciprocal lattice vectors a^* and b^* are shown. Some of the highest resolution reflections apparent by eye are circled. These are the (12, ± 4) at 34.6-Å resolution, the (11, ± 3), the (8, ± 4), and the (1, ± 5). Some higher resolution reflections have relatively low amplitudes and are thus difficult to discern by eye above the background noise but are present in the digitized, averaged data.

FIG. 5. Projection map calculated from noise-filtered Fourier transforms corrected for lattice distortions and assuming *C222* symmetry. The solid contours represent stain-excluding regions (protein density). The unit cell and its symmetry operations are shown; thick solid lines represent mirror planes, while thick dashed lines represent glide planes. Two-fold rotation axes perpendicular to the plane of the crystal are also shown (●). The width of the map corresponds to 750 Å.

average amplitude *R* factor of 0.3. Data from the four areas, corrected for lattice distortions and assuming *C222* symmetry, were merged to compute an average projection map (Fig. 5).

The unit cell contains eight areas of irregularly shaped protein density, each approximately 120×110 Å. These dimensions may be compared with those of single pol II molecules in negative stain, which range from about 110 to 150 Å in diameter (Fig. 6). Thus, we identify each of the eight areas of protein density in the unit cell as a single pol II molecule.

Views of single particles often had a V-shaped appearance (Fig. 6). The molecule was divided in two roughly equal parts by a line of negative stain down the middle. This line of stain penetrated the molecule to a depth of about 90 Å and was about 30 Å across at the surface.

DISCUSSION

The purification of pol II by immunoaffinity chromatography proves to be a general procedure. Known to be effective for the wheat germ and calf thymus enzymes (N.E.T., D. B.

FIG. 6. Electron micrographs of negatively stained single molecules of yeast pol II. (Upper) Molecules adsorbed at random to a hydrophilic electron microscope grid surface. Examples of molecules showing a stain-filled cleft are circled. The width of the micrograph corresponds to 0.65 μm . (Lower) Gallery of individual molecules at high magnification. The total width corresponds to 0.19 μm .

Aronson, and R.R.B., unpublished work), the method is applicable to yeast pol II as well. The resulting yeast enzyme contains 10 polypeptides, as reported for preparations made by conventional methods involving many more steps of fractionation. Apparently, the conventional approach did not result in the loss or adventitious association of any components as a consequence of the length of the procedure or the particular steps used. Immunoprecipitation experiments with other antibodies have led to a similar conclusion (R. Young, personal communication).

The assay for accurate initiation by yeast pol II presented here, involving complementation of a pol II-deficient extract, may be generally useful for analyzing mutant forms of the enzyme. The properties of the temperature-sensitive pol II present in the extract illustrate the importance of this type of analysis. The temperature-sensitive enzyme showed appreciable initiation activity, whereas it was virtually inert in conventional assays of ribonucleotide polymerization. There is evidently no strict correlation between initiation and polymerization activities.

The formation of two-dimensional crystals on positively charged lipid layers, previously found for *E. coli* RNA polymerase holoenzyme (23) and here demonstrated for yeast pol II, is also applicable to yeast pol I (P. Schultz, H. Celia, M. Riva, S.A.D., P. Colin, A. Sentenac, R.D.K., and P. Oudet, unpublished work). It is apparent from the projection maps calculated from the yeast pol II crystals, even without imposing symmetry, that the profiles of some molecules are opposite in hand to others. This implies that some molecules adsorbed on the lipid layer are flipped with respect to others. While this phenomenon was not observed in the *E. coli* RNA polymerase holoenzyme crystals, where the molecules interact with the lipid layer in only one orientation (23), it has been observed in two-dimensional crystals of other proteins on lipid layers (32). Features of the yeast pol II molecule such as the shape and arrangement of subunits, which are not clear in the projection map, probably due to disadvantageous orientations of the molecule on the lipid layer, will become apparent when the structure is determined in three-dimensions. The combination of biochemical and structural approaches described here, together with the genetic approaches that are available in yeast, provide a unique opportunity to explore the structure and function of the pol II enzyme.

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