Purification and Subunit Structure of Deoxyribonucleic Acid-dependent Ribonucleic Acid Polymerase III from the Mouse Plasmacytoma, MOPC 315*

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Class III DNA-dependent RNA polymerases were purified from the mouse plasmacytoma, MOPC 315. RNA polymerases III, and III, were solubilized from a whole cell extract and resolved by chromatography on DEAE-Sephadex. Chromatography on DEAE-cellulose, DEAE-Sephadex, CM-Sephadex, and phosphocellulose ion exchange resins and sedimentation in sucrose density gradients yielded chromatographically homogeneous Enzymes III, and III, which were purified approximately 22,000 and 53,000-fold, respectively, relative to whole cell extracts. The specific activity of these enzymes was comparable to that reported for other purified eukaryotic RNA polymerases. Sucrose gradient sedimentation analysis suggested a molecular weight of approximately 650,000 for each of the class III enzymes.

The subunit compositions of chromatographically purified RNA polymerases III, and III, were analyzed by polyacrylamide gel electrophoresis under denaturing conditions. RNA polymerase III, contained subunits with molecular weights of 155,000 (IIIa), 138,000 (IIIb), 89,000 (IIIc), 70,000 (IIId), 53,000 (IIIe1), 49,000 (IIIe2), 41,000 (IIIf), 32,000 (IIIg), 29,000 (IIIg2), 19,000 (IIIf). RNA polymerase III, subunits were identical with those of Enzyme III, except for the replacement of subunit IIIg with a slightly larger subunit IIIgB (Mr = 33,000). Molar ratios were close to unity for all subunits except for IIIf, which was present in stoichiometric excess, yielding a composite molecular weight of approximately 695,000.

Analysis of purified RNA polymerases III, and III, by polyacrylamide gel electrophoresis under non-denaturing conditions revealed, in each instance, two major protein bands. Subsequent analyses of the two electrophoretic forms of Enzyme III, failed to reveal any structural differences since each form contained subunits IIIa to i in the same proportions as found in the unfraccionated phosphocellulose enzyme.

The present data have been used to estimate the cellular concentrations of RNA polymerase III molecules. MOPC 315 cells have approximately $3 \times 10^6$ molecules/cell, while the concentrations in other cell types are estimated to be as much as 50-fold lower. However, the specific activities and subunit compositions of the enzymes purified from mouse plasmacytoma cells and from normal tissues appear similar. Thus, fluctuations in the cellular levels of RNA polymerase III activity may, in part, result from changes in the cellular concentration of RNA polymerase III molecules. The data are discussed in terms of the regulation of transfer RNA and 5 S RNA synthesis and cell growth rate by RNA polymerase III.

DNA-dependent RNA polymerase III represents one of the three principal classes of nuclear RNA polymerase found in eukaryotic cells (1). RNA polymerase III was originally distinguished from RNA polymerases I and II on the basis of its distinct catalytic properties and its elution from DEAE-Sephadex at high salt concentrations (1), features which now appear common to most or all class III enzymes (1-13). RNA polymerase III usually accounts for a small proportion of the total RNA polymerase activity and has been detected, using appropriate analytical methods, in all tissues examined (2). Chromatographically heterogeneous forms of these enzymes have been described in mouse plasmacytomas (2), mouse liver and spleen tissues (2), human peripheral lymphocytes (3), calf thymus (2), rat liver (9), and Xenopus laevis liver tissue. In contrast, only one class III enzyme has been detected in lower eukaryotes and this enzyme appears resistant to high concentrations of a-amanitin (10-13). There has been no report of physical, structural, or functional differences among the heterogeneous class III enzymes.
mediated via changes in enzyme concentration. These studies also provide evidence that fluctuations in RNA polymerase III activity might provide insights into the regulation of tRNA and 5 S RNA synthesis and cell growth rates.

To investigate these problems, we have chosen the mouse plasmacytoma, MOPC 315, a rapidly growing malignant cell line. These cells contain high levels of RNA polymerase III (2), probably reflecting a high level of tRNA and 5 S RNA synthesis characteristic of a rapid rate of cellular proliferation (13). This paper reports the purification and subunit structures of the class III enzymes from MOPC 315 cells, which have permitted paper reports the purification and subunit structures of the class III enzymes from MOPC 315 cells, which have permitted the heterogeneity of these particles to be studied. These cells contain high levels of RNA polymerase III (2), which might provide insights into the regulation of tRNA and 5 S RNA synthesis and cell growth rates.

EXPERIMENTAL PROCEDURES

Cells

MOPC 315 solid tumors were obtained as described previously (2).

Biochemicals

Unlabeled nucleotide triphosphates were obtained from P-L Biochemicals; [3H]UTP from New England Nuclear; crystalline bovine serum albumin from Pentex; acrylamide, bisacrylamide, and tetramethylthielenediamine (TEMED) from both Eastman and Bio-Rad Laboratories; a-amamin from Henley and Co.; calf thymus DNA type I, Nonidet P-40, PMSF, and iPr,P-F from Sigma; and [d(A-T)] from Miles.

Ion Exchange Resins

DEAE-cellulose (Whatman DE52) and phosphocellulose (Whatman P-11) obtained from Pharmacia were prepared as described previously (2).

Conductivity, DNA, and Protein Measurements

Assays were performed in a final volume of 25 μl as described previously (3). Calf thymus DNA or (d(A-T))₆ was used as template at a final concentration of 100 or 50 μg/ml, respectively. One unit of activity represents incorporation of 1 pmol of UMP into RNA in 20 min at 35°. The supernatant was centrifuged for 20 min at 18,000 rpm in a Sorval SS-34 rotor. The supernatant was centrifuged for 120 min at 50,000 rpm in a Spincro type Ti-60 rotor. The resultant 0.32 m ammonium sulfate supernatant fractions (F1) were diluted to 0.1 m ammonium sulfate with Buffer A and aggregated chromatin was pelleted by centrifugation at 50 min at 50,000 rpm. To the 0.1 m ammonium sulfate supernatant fractions (F2), solid ammonium sulfate (mann enzyme grade) was added to saturating levels (0.42 g/ml). The precipitate was pelleted by centrifugation for 70 min at 35,000 rpm in a Spincro type 42 rotor. The ammonium sulfate pellets were resuspended by addition of Buffer B (4.6 mg of bovine serum albumin/ml of bed volume). The resins were washed with 2 column volumes of the equilibration buffer and eluted with a linear gradient of 0.05 to 0.25 M ammonium sulfate in Buffer B. The total gradient size was equivalent to 4 column volumes and fractions equivalent to 2.5% of the gradient volume were collected. Following this, the remainder of the adsorbed activity was eluted with Buffer B containing 0.5 m ammonium sulfate. Appropriate column fractions containing class III RNA polymerases were combined and assayed for activity and protein content.

DEAE-Sephadex Chromatography—Fraction F4 was then subsequently diluted with Buffer B to a final salt concentration of 0.03 m ammonium sulfate. This sample was loaded onto a DEAE-cellulose column equilibrated with Buffer B containing 0.05 m ammonium sulfate (1 to 1.5 mg of protein/ml of bed volume). The resin was washed with 2 column volumes of the equilibration buffer and eluted with a linear gradient of 0.05 to 0.25 M ammonium sulfate in Buffer B. The gradient size was 3 column volumes and fractions corresponding to 2.5% of the gradient volume were collected. Fractions containing RNA polymerase III were combined and assayed for enzyme activity and protein content. Bovine serum albumin was then added (0.5 mg/ml) to help stabilize enzyme activity and the solution stored at -80°. Similarly, fractions containing RNA polymerase IIIb were combined, bovine serum albumin was added, and the solution stored at -80°.

First Phosphocellulose Chromatography—The combined fractions from DEAE-cellulose chromatography were directly loaded onto a DEAE-Sephadex column equilibrated with Buffer B containing 0.1 m ammonium sulfate (1 to 1.5 mg of protein/ml of bed volume). The resin was washed with 1 column volume of equilibration buffer and 1 column volume of Buffer B containing 0.08 m ammonium sulfate and eluted with a linear gradient of 0.02 to 0.4 m ammonium sulfate in Buffer B. The gradient size was 5 column volumes and fractions equivalent to 2.5% of the gradient volume were collected. Fractions containing RNA polymerase III were combined and assayed for enzyme activity. Bovine serum albumin was then added (0.5 mg/ml) to help stabilize enzyme activity and the solution stored at -80°. Similarly, fractions containing RNA polymerase IIIb were combined, bovine serum albumin was added, and the solution stored at -80°.

Purification Methods

RNA Polymerase Solubilization—RNA polymerase was solubilized from whole cells by a modification of a previously published procedure (2). MOPC 315 tumors (100 g) were first homogenized (15 strokes) in 3 vol of Buffer A (0.15 m NaCl, 0.05 m Tris-HCl (pH 7.9, 2.5 mM iPr,P-F, 25 mM dithioerythritol, 5 mM MgCl₂, 1 mM PMFSF, and 1 mM iPr,P,F) and passed through a double-layer of cheesecloth. The suspension was adjusted to 0.32 M ammonium sulfate and the viscous supernatant pellets were sonicated and resuspended previously (2). This solution was centrifuged for 20 min at 18,000 rpm in a Sorval SS-34 rotor. The supernatants were centrifuged for 120 min at 50,000 rpm in a Spincro type Ti-60 rotor. The resultant 0.32 M ammonium sulfate supernatant fractions (F1) were diluted to 0.1 m ammonium sulfate with Buffer A and aggregated chromatin was pelleted by centrifugation for 50 min at 50,000 rpm. To the 0.1 m ammonium sulfate supernatant fractions (F2), solid ammonium sulfate (mann enzyme grade) was added to saturating levels (0.42 g/ml). The precipitate was pelleted by centrifugation for 70 min at 35,000 rpm in a Spincro type 42 rotor. The ammonium sulfate pellets were resuspended by addition of Buffer B (4.6 mg of bovine serum albumin/ml of bed volume). The resins were washed with 2 column volumes of the equilibration buffer and eluted with a linear gradient of 0.05 to 0.25 M ammonium sulfate in Buffer B. The total gradient size was equivalent to 4 column volumes and fractions equivalent to 2.5% of the gradient volume were collected. Following this, the remainder of the adsorbed activity was eluted with Buffer B containing 0.5 m ammonium sulfate. Appropriate column fractions containing class III RNA polymerases were combined and assayed for activity and protein content.

First Phosphocellulose Chromatography—The combined fractions from DEAE-cellulose chromatography were directly loaded onto a DEAE-Sephadex column equilibrated with Buffer B containing 0.1 m ammonium sulfate (1 to 1.5 mg of protein/ml of bed volume). The resin was washed with 1 column volume of equilibration buffer and 1 column volume of Buffer B containing 0.08 m ammonium sulfate and eluted with a linear gradient of 0.02 to 0.4 m ammonium sulfate in Buffer B. The gradient size was 5 column volumes and fractions equivalent to 2.5% of the gradient volume were collected. Fractions containing RNA polymerase III were combined and assayed for enzyme activity. Bovine serum albumin was then added (0.5 mg/ml) to help stabilize enzyme activity and the solution stored at -80°. Similarly, fractions containing RNA polymerase IIIb were combined, bovine serum albumin was added, and the solution stored at -80°. The following procedures were used to purify further the chromatographically separated RNA polymerase III and IIIb.

CM-Sephadex Chromatography—The combined fractions from DEAE-Sephadex chromatography (i.e. containing either III, or IIIb) were thawed and dialyzed against 10 volumes of Buffer B to a final concentration of 0.04 m ammonium sulfate. The dialyzed preparation was loaded onto a CM-Sephadex (Whatman CM-52, packed homogenization column) equilibrated with Buffer C (Buffer B plus 0.5 mg/ml of bovine serum albumin) containing 0.05 m ammonium sulfate. The column was washed with 2 column volumes of Buffer C containing 0.03 m ammonium sulfate. The enzyme was eluted with a linear gradient of 0.03 to 0.25 m ammonium sulfate in Buffer C. The gradient size was 3 column volumes and fractions equivalent to 3% of the gradient volume were collected. During the loading and washing of the column and during gradient elution of the column the flow rates were, respectively, 0.06 and 0.033 ml/min/ml of bed volume. Fractions containing RNA polymerase III were pooled and reasayed for enzyme activity with calf thymus DNA.
stained in 10% (v/v) acetic acid, 50% (v/v) ethanol, and 0.14 (w/v) ammonium sulfate. Fractions of 0.2 ml were collected at a flow rate of 0.5 ml/min and assayed for activity with calf thymus DNA.

**Sucrose Density Gradients**—The peak activity fractions from the first phosphocellulose column were individually layered directly onto 5 to 20% sucrose density gradients (4.4 ml) which were prepared and then used exactly as described previously (17). Unless otherwise specified, fraction volumes of 0.22 ml were collected and assayed for activity with calf thymus DNA. Although the purification effected by this procedure is not evident from the data in Table II, omission of this step resulted in an increased background of low molecular weight polypeptides (<80,000) in the final phosphocellulose enzyme preparation (below).

**Second Phosphocellulose Chromatography**—The peak activity fractions from the sucrose gradients were individually loaded onto a second phosphocellulose column equilibrated with Buffer B containing 0.05 M ammonium sulfate (100,000 to 150,000 units of activity/ml of bed volume). The resin was washed with Buffer B containing 0.05 M ammonium sulfate and eluted with a 10 column volume linear gradient of 0.05 to 0.12 M sodium sulfate in Buffer B. Fraction volumes equivalent to 3% of the gradient volume were collected and individually assayed for RNA polymerase activity and protein content. These individual fractions were then stored at -80°C.

**Polyacrylamide Gel Electrophoresis**—Phosphocellulose gradient (containing RNA polymerases II and III) were subjected to electrophoresis on polyacrylamide gels in cylindrical tubes under both denaturing and non-denaturing conditions as described previously (17).

The denaturing sodium dodecyl sulfate gel system is a modification of Laemmli's procedure (21). These gels were fixed in 25% (v/v) acetic acid and then photographed after removal of the stain. The dye had stacked and entered the gel, and then the current was turned off. The gel slab (25 x 9 x 0.1 cm) was then washed for 3 hours at 25°C and stained for 4 hours at 25°C, using the solutions described above. The gel slab was then destained with several washes of 10% (v/v) acetic acid, 50% (v/v) ethanol, and 0.14 (w/v) ammonium persulfate. The gel tubes were then placed on a Gilford linear transport device for 10 to 20 hours at 50°C. Gels were destained of Laemmli's procedure (21). These gels were fixed in 12% (w/v) glycerol. O.175 M Tris-HCl (pH 6.8), 0.15 M dithioerythritol, 0.06% (v/v) TEMED, and 0.08% (w/v) ammonium persulfate. Samples were subjected to electrophoresis at 1 mA/gel until the dye had stacked and entered the gel, and then the current was increased to 1.5 mA/gel until the dye front reached the bottom of the gel. These gels were destained and stained bands were scanned with a Gilford linear transport device as described previously (17).

**Elongation under non-denaturing conditions** was performed on polyacrylamide gels which were constructed according to the method of Maizel (22). Fresh dithioerythritol and bromphenol blue were added to each sample to final concentrations of 20 mM and 0.005%, respectively. This solution was then layered over a 1.3 ml polyacrylamide gel (6.5 x 0.3 cm) containing 5% acrylamide, 0.13% bisacrylamide, 25% (v/v) glycerol, 0.75% (w/v) Tris-HCl (pH 8.9), 0.15% dithioerythritol, 0.06% (v/v) TEMED, and 0.08% (w/v) ammonium persulfate. The gel tubes were then filled with buffer electrode (5 mM Trisazma base and 38 mM glycerine). Samples were subjected to electrophoresis at 1 mA/ml until the dye had stacked and entered the gel, and then the current was increased to 1.5 mA/ml until the dye front reached the bottom of the gel. These gels were either stained for protein or sliced. In the latter case, slices were subjected to electrophoresis under denaturing conditions as described previously (17).

In addition, class III RNA polymerases were subjected to electrophoresis under denaturing conditions on a high resolution polyacrylamide gel slab (35 x 9 x 0.1 cm) using a procedure similar to that described by Studier (23). The resolving gel, 24 cm in length, contained a linear 6 to 11% acrylamide gradient. A 3% acrylamide stacking gel 1 cm in length, was polymerized above the gradient resolving gel. Electrophoresis of the samples through the stacking gel was carried out at 110 volts (constant voltage) for 90 min and then the voltage was then raised to 150 volts. The stacking dye had completely run through the resolving gel (i.e., approximately 12 hours total). The gel slab was fixed for 3 hours at 25°C and stained for 1 hours at 25°C, using the solutions described above. The gel slab was then destained with several washes of 10% (v/v) acetic acid and then photographed after removal of the stacking gel. Unless otherwise stated, all reagents used for polyacrylamide gel electrophoresis were those obtained from Bio-Rad Laboratories.

**RESULTS**

**Solubilization, Chromatography, and Sucrose Gradient Sedimentation**—Solubilized extract (F4), containing class I, II, and III RNA polymerases, was obtained as described under "Experimental Procedures." As summarized in the upper part of Table I, these prechromatographic procedures remove 65% of the contaminating protein in Fraction F1, with no detectable loss in total RNA polymerase activity. The apparent increase in activity during these initial purification steps may be due to removal of inhibitory proteins.

RNA polymerase Fraction F4 was subjected to the chromatographic and sedimentation procedures described under "Experimental Procedures." Chromatography of Fraction F4 on DEAE-cellulose revealed two major peaks of activity (Fig. 1). The activity eluting at 0.07 to 0.13 M ammonium sulfate represents a mixture of class I and II RNA polymerases as determined by its insensitivity to low concentrations (0.5 µg/ml) of α-amanitin (2) and by its elution behavior on DEAE-Sephadex (see below). The activity eluting from DEAE-cellulose at 0.16 to 0.30 M ammonium sulfate was completely sensitive to low concentrations of α-amanitin and therefore represents exclusively RNA polymerase II (2).

The peak DEAE-cellulose fractions, insensitive to low levels of α-amanitin, were combined and subjected to DEAE-Sephadex chromatography (Fig. 2). This procedure resolves RNA polymerase I and the individual class III RNA polymerase...
and with calf thymus DNA (O---O); -, ammonium sulfate concentration; protein (mg/ml).

Fractions 8 to 18 were combined and assayed for protein content. The data summarized in Table I (except for "Total Activity") are based on values for these 11 fractions. Activity was measured with calf thymus DNA in the absence (O---O) or presence (G---G) of 0.5 µg/ml of α-amanitin; —, ammonium sulfate concentration; ---, absorbance at 280 nm.

Overall Purification and Recovery—The purification of the individual class III enzymes after DEAE-Sephadex chromatography is summarized in Table II. Specific activities shown for the final phosphocellulose enzymes are the average from all enzyme-containing fractions. Specific activities measured across the peak phosphocellulose fractions averaged 315 units/µg of protein with calf thymus DNA and 5,450 units/µg of protein with [d(A-T)]₅ as templates (see below). Due to the high concentration (0.1 M) of ammonium sulfate in the input sample, the majority of the enzyme I activity was not adsorbed (see legend to Fig. 2). As described previously (2), MOPC 315 cells contain two chromatographically distinct forms of RNA polymerase III, designated III₁ and III₂. As summarized in the lower part of Table I, the DEAE-cellulose and the DEAE-Sephadex chromatographic steps yield an approximately 70-fold purification with greater than 90% recovery of class III activity.

The following procedures were used to purify further the chromatographically separated RNA polymerases III₁ and III₂. Since these two enzymes display similar behavior on CM-Sephadex and phosphocellulose chromatography, as well as on sucrose gradient sedimentation in the presence of 0.08 M ammonium sulfate, only the purification of RNA polymerase III₂ will be described.

DEAE-Sephadex fractions containing 349,000 units of RNA polymerase III₂ activity (Fig. 2) were combined and subjected to chromatography on a CM-Sephadex column (12 × 2.5 cm) as described under "Experimental Procedures." Less than 5% of the input activity appeared in the breakthrough fractions. The activity which bound to the column was eluted in a single symmetrical peak at an ammonium sulfate concentration of 0.10 M. A total of 160,000 units of activity were recovered. The peak fractions (containing 135,000 units) were pooled and loaded onto a phosphocellulose column (1.8 × 1.2 cm) as described under "Experimental Procedures" (see "First Phosphocellulose Chromatography"). All of the activity was adsorbed to the column and eluted in a single sharp peak with a maximal enzyme concentration (peak tube) of 120,000 units/ml. A total of 105,000 units of activity were recovered. Those fractions which contained enzyme concentrations in excess of 30,000 units/ml were subjected individually to sucrose gradient sedimentation at an ammonium sulfate concentration of 0.08 M, as described under "Experimental Procedures." A total of 95,000 units were loaded onto the sucrose gradient and the apparent yield of activity in this step was 103%. The sedimentation profile was similar to that observed in experiments described below. The final purification step was adsorption of the enzyme from the peak sucrose gradient fractions to a second phosphocellulose column and elution with a linear salt gradient (Fig. 3).
to 3 and in the text, not all of the activity recovered at each step was collected for subsequent purification procedures. Had no activity been discarded at each of these stages, an overall yield of 27% or 39% should have been attained, as measured with calf thymus DNA or [d(A-T)]$_4$, respectively. These recoveries of activity were obtained by minimizing the number

and degree of dilutions of enzyme solutions, by the inclusion of bovine serum albumin in the buffers used for CM-Sephadex and the first phosphocellulose chromatography, and by the addition of bovine serum albumin and Nonidet P-40 to sucrose density gradients (17).

Properties—RNA polymerases III$_A$ and III$_B$ have many similar properties which distinguish them from the corresponding class I and class II enzymes. These include (a) biphasic salt activation profiles with native DNA templates (2); (b) distinct chromatographic behavior on DEAE-cellulose (elution at low ionic strength) versus DEAE-Sephadex (elution at high ionic strength) (Figs. 1 and 2); (c) sensitivity to high concentrations of a-amanitin (50% inhibition at 20 µg/ml) (2); and (d) increased activity (11- to 16-fold) with [d(A-T)]$_4$ as template, relative to native DNA (Figs. 2 and 3).

Thus far only minor differences in the properties of RNA polymerase III$_A$ and III$_B$ have been detected. As shown above, the enzymes show distinct chromatographic properties on DEAE-Sephadex (Fig. 2). In addition, RNA polymerase III$_A$ can be distinguished from RNA polymerase III$_B$ by sucrose gradient sedimentation at intermediate ionic strengths (Fig. 4). In the presence of 0.125 M ammonium sulfate, Enzyme III$_B$ sediments as a single peak of activity, while Enzyme III$_B$ sediments as a double peak of activity (Fig. 4, Panels B and E, respectively). Similar results were observed at 0.1 M ammonium sulfate.
Fig. 5. Subunit pattern of RNA polymerase III, according to position in the phosphocellulose column gradient. RNA polymerase III, (48,000 units of activity) was purified as described in this report. Equivalent volumes of Fractions 9 to 16 (containing 450 to 2,300 units of activity) from the second phosphocellulose chromatography (Fig. 3) were subjected to electrophoresis in 10% polyacrylamide gels containing sodium dodecyl sulfate as described under "Experimental Procedures."

Sulfate both enzymes sediment as single peaks of activity as shown in Panels C and F (Fig. 4). However, at concentrations of ammonium sulfate lower than 0.1 m (Fig. 4, Panels A and D), both enzymes display heterogeneous peaks of activity. These data are consistent with the idea that Enzyme IIIa aggrates at both low and intermediate ionic strengths, whereas Enzyme IIIb does so only at low ionic strengths. Although these enzymes have been purified by chromatography on two cation exchange columns and on two strong anion exchange columns prior to sedimentation on sucrose gradients, the possibility remains that a contaminating substance (e.g. a nucleic acid) may be responsible for the characteristic sedimentation properties of RNA polymerases IIIa and IIIb.

Polyacrylamide Gel Electrophoresis under Denaturing Conditions-Individual phosphocellulose gradient fractions, containing RNA polymerase IIIa activity, were subjected to electrophoresis in the presence of sodium dodecyl sulfate (Fig. 5). The 10 polypeptides designated IIIa,b,c,d,e1,e2,f,g,h, and i, in order of decreasing molecular weight, are regarded as putative subunits based on the following observations. First, the mass of each of these polypeptides is directly proportional to the enzyme activity present in each phosphocellulose gradient fraction (Fig. 5). In contrast, this relationship does not hold for the additional polypeptides which are apparent in the various gradient fractions, with the possible exception of the 24,000- and 22,000-dalton polypeptides which migrate between subunits IIIh and IIIi (see also below). Second, the unlabeled polypeptides in Fig. 8 are not consistently detected in various enzyme preparations and account for less than 3% of the total protein present in peak activity fractions. Third, polypeptides IIIa to h are present in approximately equimolar amounts in each gradient fraction. Although an accurate molar ratio determination could not be obtained for subunit IIIi, this band was observed in stoichiometric excess and may thus represent more than one polypeptide (see below). The molecular weights and molar ratios of RNA polymerase IIIa, subunits are summarized in the second and fourth columns of Table III, respectively.

<table>
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<th>Subunit</th>
<th>Molecular weight</th>
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* See text for a discussion of the heterogeneity of subunit i.

Approximately unity (Table III). However, heterogeneity in polypeptide IIIa has also been observed in the present studies, when the enzyme is subjected to electrophoresis in a high resolution polyacrylamide gel slab (Fig. 8). The molar ratios of the individual heterogeneous IIIa polypeptides vary in different enzyme preparations, but their sum is approximately unity. The basis for the heterogeneity in subunit IIIa is not known.

Table III

Subunit Structure of Mouse Plasmacytoma RNA Polymerase III

Class III RNA polymerase subunit compositions were examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with enzymes obtained from phosphocellulose gradient fractions and with the electrophoretic forms of III, observed on non-denaturing polyacrylamide gels. Molecular weights were averaged from several preparations and varied from the values shown by less than 5% in the case of subunits IIIa and IIIb and by less than 10% in the case of subunits IIIc to i. Molar ratios were normalized to subunit IIIb. Molar ratios for the indicated subunits, measured across phosphocellulose gradient fractions, were fairly constant, varying less than 20% from the average values shown (Fig. 5). Molar ratios for electrophoretic forms III,-1 and III,-2 were obtained from Fig. 7. In some instances (see Fig. 7) additional staining material was apparent in the region between subunit IIIb and the dye front. Thus, molar ratio determinations for subunit IIIb are not as accurate as for the remaining subunits. The electrophoretic forms of Enzyme IIIa displayed a heterogeneous shoulder of staining material which migrated slightly faster than subunit IIIb. This material was not included in molar ratio determinations for subunit IIIb and has not been further examined. In addition, the indicated molar ratios must be regarded as approximate since the staining intensity of individual proteins may not always be proportional to molecular weights (cf. Ref. 26).
Polyacrylamide Gel Electrophoresis under Nondenaturing Conditions—Highly purified RNA polymerase III, (phosphocellulose gradient fraction) was analyzed by polyacrylamide gel electrophoresis under nondenaturing conditions. When these gels were stained for protein, two major bands (designated III,-1 and III,-2) were routinely observed, and one minor diffuse band was occasionally detected (Fig. 6). Greater than 95% of the protein stain was associated with these bands. The migration of these bands and their relative intensities were somewhat variable in different experiments, yielding two patterns as shown in Fig. 6 (compare Gels 1 and 2). The cause of this variability is not clear, but samples containing high concentrations of enzyme seemed to yield the pattern shown in Gel 1 (Fig. 6), while less concentrated samples yielded the pattern shown in Gel 2 (Fig. 6). However, other factors such as minor differences in the salt concentration of the samples may contribute to this variability. No activity measurements (see Ref. 17) were attempted on the electrophoretically separated protein bands.

Polyacrylamide Gel Electrophoresis under Denaturing Conditions Following Electrophoresis under Nondenaturing Conditions—The subunit compositions of electrophoretic forms III,-1 and III,-2 have been determined. An unstained polyacrylamide gel, containing 6 times more sample protein than Gel 1 (Fig. 6), was divided into 1-mm wide slices and the protein in the individual slices was subjected to electrophoresis under denaturing conditions as described previously (17). Panel A in Fig. 7 shows the subunit composition of the phosphocellulose enzyme prior to electrophoresis under nondenaturing conditions. Panels B and C in Fig. 7 show the polypeptide compositions of electrophoretic forms III,-1 and III,-2, respectively. The subunit compositions of electrophoretic forms III,-1 and III,-2 were identical and, except for a shoulder of staining material migrating slightly faster than subunit III,-, they were the same as that of the phosphocellulose enzyme. Subunit molecular weights and molar ratios are summarized in Table III.

Fig. 6. Polyacrylamide gel electrophoresis under nondenaturing conditions. Samples of RNA polymerase III, and III, from the second phosphocellulose column fractions were subjected to electrophoresis on 5% polyacrylamide gels under nondenaturing conditions as described under "Experimental Procedures." Gel 1, electrophoresis of RNA polymerase III, (900 units); Gel 2, electrophoresis of RNA polymerase III, (350 units); and Gel 3, electrophoresis of RNA polymerase III, (400 units). Polyacrylamide Gels 2 and 3 were run identically and in平行.

The enzyme preparation analyzed in this experiment (Fig. 7) also appeared to contain two polypeptide components between polypeptides III,-1 and III,- (cf. Fig. 5). Although the polypeptide bands were diffuse (Panels B and C), these two components appeared to remain associated with electrophoretic forms III,-1 and III,-2. As noted above, however, these components are not readily detected in all RNA polymerase III preparations.

A small amount of protein was recovered from slices corresponding to the minor diffuse band in Gel 1 of Fig. 6. With the probable exception of polypeptides III,-1 and III,-2, this material contained all the subunits present in the phosphocellulose enzyme.
enzyme and may thus represent a partially dissociated RNA polymerase III molecule.

Electrophoretic Comparison of RNA Polymerases IIIa and IIIb—Electrophoresis of RNA polymerase IIIb under non-denaturing conditions revealed two major protein bands similar to the results obtained with Enzyme IIIa (Fig. 6). The subunit compositions of electrophoretic forms IIIa-1 and IIIb-2 were not investigated.

Electrophoresis of individual phosphocellulose gradient fractions containing RNA polymerase IIIa activity revealed a subunit pattern similar to the one obtained with Enzyme IIIa.3 To resolve minor differences in the subunit compositions between RNA polymerases IIIa and IIIb, these enzymes were subjected to electrophoresis individually and in combination on a 25-cm polyacrylamide gel slab under denaturing conditions (Fig. 8). These data clearly illustrate that RNA polymerases IIIa and IIIb differ only in one subunit. Except for a 32,000 dalton subunit IIIg which is unique to Enzyme IIIa, and a 33,000 dalton subunit IIIg which is unique to Enzyme IIIb, the subunit molecular weights in Enzyme IIIa appear identical with those in Enzyme IIIb (Table III). The molar ratios of the subunits in Enzyme IIIb are similar to those for the analogous subunits in Enzyme IIIa.4 The electrophoretic system used here (Fig. 8) resolves polypeptide IIIf (Fig. 5) into two components as observed previously (Ref. 16, see also Footnote 4). This electrophoretic system also resolves subunit IIIi into several polypeptides. Although subunit IIIi appears to be present in stoichiometric excess after electrophoresis under non-denaturing conditions (Fig. 7), it is not clear which of the low molecular weight polypeptides observed in Fig. 8 remain associated with the enzyme under these conditions.

**DISCUSSION**

Purification, Structure, and Heterogeneity of RNA Polymerases IIIa and IIIb—Two chromatographic forms of RNA polymerase III are present in the mouse plasmacytoma MOPC 315. Previous studies have shown the presence of RNA polymerase III activity in both cytoplasmic and nuclear fractions following cellular disruption and fractionation (2, 5, 8, 9). We have, therefore, purified the MOPC class III enzymes from whole cells in order to study the total cellular population of these molecules. RNA polymerases IIIa and IIIb were resolved by chromatography on DEAE-Sephadex and purified by ion exchange chromatography and sucrose gradient sedimentation. Relative to whole cell extracts the overall purifications were 22,000- and 53,000-fold, respectively, for enzymes IIIa and IIIb. Chromatographically homogeneous RNA polymerases IIIa and IIIb each contains at least 10 putative subunits designated IIIa,b,c,d,e1,e2,f,g,h, and i. That these RNA polymerase III-associated polypeptides represent enzyme subunits is suggested by the following observations: (a) the ratio of the amount of each polypeptide to the amount of enzyme activity is approximately constant for individual phosphocellulose gradient fractions; (b) the molar ratios of these polypeptides are approximately unity, with the exception of polypeptide IIIi which is present in a higher but constant molar ratio; (c) the molecular weight of RNA polymerase III calculated from the molecular weights and molar ratios of the individual polypeptides (695,000) is compatible with that estimated from sucrose density gradient sedimentation (650,000); (d) polypeptides IIIa to i co-sediment with RNA polymerase III activity upon sucrose density gradient sedimentation and they remain associated with the major protein bands when RNA polymerase III is subjected to electrophoresis under non-denaturing conditions; and (e) the murine and amphibian class III enzymes contain analogous polypeptides of the same or similar size (16), even though these enzymes are from grossly different cell types.

The subunit compositions of RNA polymerases IIIa and IIIb are very similar, differing only in subunit IIIg which is slightly smaller in Enzyme IIIb (IIIg, 32,000 daltons) than in Enzyme IIIa (IIIg, 33,000 daltons). As reported previously (2), no evidence for interconversion of these enzyme forms could be found since they maintain their distinctive properties upon rechromatography on DEAE-Sephadex. The presence of serine protease inhibitors (i.e. PMSF and iPr,P-F) during enzyme isolation and purification did not alter the subunit patterns of the purified enzymes. The general similarity between the subunit structures of Enzymes IIIa and IIIb correlates with the similarities in their catalytic properties and α-amanitin sensitivities. However, the minor structural difference between subunits IIIg1 and IIIg2 may be responsible for differences in
the sedimentation properties and DEAE-Sephadex elution positions of Enzymes III, I, and II, and might also reflect functional differences. Chromatographically purified RNA polymerases III, I, and II were each resolved into two electrophoretic forms. The subunit compositions of the electrophoretic forms of RNA polymerase III, I, and II were indistinguishable. Therefore, the electrophoretic forms of RNA polymerase III may differ primarily in the charge of a specific subunit(s), which would not be detected by electrophoresis in the presence of sodium dodecyl sulfate. Alternatively, these electrophoretic forms could reflect different states of aggregation of the enzyme or other minor structural differences not detectable in the present analytical systems.

Alterations in RNA Polymerase Activity—Class III RNA polymerases have been shown to synthesize tRNA and 5 S RNA in mouse plasmacytoma cell nuclei (14). These enzymes have also been shown to synthesize several distinct, low molecular weight viral RNAs in adenovirus 2-infected KB cells (24) which, like plasmacytoma cells, contain two chromatographic forms of RNA polymerase III (25). These observations and the structural studies presented here suggest the possibility of functional differences between RNA polymerases III, I, and II. Although the basis and significance of the distinct electrophoretic forms of each class III enzyme remain unclear, electrophoretic variants might represent distinct functional states or regulatory modifications of a single enzyme.

Previous studies have shown both increased rates of tRNA and 5 S RNA synthesis (15) and increased cellular levels of solubilized RNA polymerase III activity in more rapidly growing cell types (2, 3, see introduction to the text). In an attempt to distinguish whether increased enzyme activities result from RNA polymerase modifications or from increased enzyme concentrations, the purified class III RNA polymerases from several cell types have been compared. The intrinsic specific activity of the MOPC class III enzymes (Table II) is similar to that observed for RNA polymerase III from both Xenopus laevis oocytes and from the posterior silk gland of Bombyx mori and is comparable to the specific activities reported for other purified eukaryotic RNA polymerases (17, 18, 20-29). Furthermore, the subunit compositions of the murine and the amphibian class III enzymes are remarkably similar (15). Thus, as reported previously for RNA polymerase I (17), the variable levels of RNA polymerase III activity in different cell types (2) may reflect primarily variations in the cellular concentrations of RNA polymerase III.

From the cellular levels of RNA polymerase III activity (2) and from the specific activities of the purified enzymes it can be estimated that plasmacytoma cells contain approximately $3 \times 10^4$ molecules of RNA polymerase III. Assuming a combined value of $3,000$ to $17,000$ genes coding for 5 S and tRNAs in mammalian cells (30-32) and that each active gene could accommodate approximately two RNA polymerases (cf. Ref. 33), it appears that plasmacytoma cells contain sufficient class III enzyme molecules to saturate these genes. In contrast, less active cell types such as liver cells (2) or peripheral lymphocytes (3) contain up to 50-fold less RNA polymerase III than plasmacytoma cells and the enzymes may be limiting in these situations.

These considerations suggest a coarse level of regulation of the cellular rates of tRNA and 5 S RNA synthesis via alterations of cellular enzyme III concentrations. Such alterations in enzyme levels may, however, reflect long term adaptive changes and do not rule out the possible involvement of other enzyme regulatory factors, which may have escaped detection by the nonspecific RNA polymerase assay conditions used. Furthermore, since distinct tRNA (34) and 5 S RNA (35) genes appear to be differentially transcribed, additional components which regulate enzyme activity may be anticipated. The complex subunit structure of the class III enzymes suggests the possibility of RNA polymerase III interactions with a variety of distinct cellular components. These interactions may serve to regulate the activity of these enzymes and to integrate this regulation with other cellular processes. Furthermore, the class III enzymes may be regulated independently of the class I and II enzymes since each enzyme class is comprised in large part of distinct polypeptides (16).

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