Isolation and Partial Characterization of the Multiple Forms of Deoxyribonucleic Acid-dependent Ribonucleic Acid Polymerase in the Mouse Myeloma, MOPC 315*

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SUMMARY

RNA polymerase has been solubilized from whole cell homogenates of the mouse myeloma tumor, MOPC 315. Four forms of the solubilized enzyme have been isolated by ion exchange chromatography and designated I, II, III*, and IIIβ. The enzymes appear analogous to the Class I, II, and III RNA polymerases defined in other eukaryotic systems. Ionic strength optima, metal ion effects, α-amanitin sensitivities, and relative activities with poly[d(A-T)] and calf thymus DNA templates are different for Class I, II, and III RNA polymerases, but are identical for III* and IIIβ. For the Class I and Class II enzymes ionic strength optima occur, respectively, at 0.05 and 0.09 M ammonium sulfate with either calf thymus DNA or poly[d(A-T)] as template. By contrast, the ionic strength activation profiles for Class III RNA polymerases with calf thymus DNA template are biphasic with optima at 0.055 and 0.17 M ammonium sulfate; but with poly[d(A-T)] the profiles are monophasic with optima at 0.055 M. Although RNA polymerase I is completely resistant to α-amanitin at concentrations up to 400 μg per ml, RNA polymerases II and III can both be inhibited completely by the toxin. However, the sensitivity of RNA polymerase II (50% inhibition at 0.025 μg per ml) is much greater than that of the Class III RNA polymerases (50% inhibition at 20 μg per ml). Furthermore, the poly[d(A-T)] to calf thymus DNA activity ratios are considerably greater for the Class III RNA polymerases than for RNA polymerases I and II. By measuring RNA polymerase activity in DEAE-Sephadex fractions at α-amanitin concentrations just sufficient to inhibit RNA polymerase II and in the presence of poly[d(A-T)] RNA polymerase III activity has been detected in all tissues examined.

When the amounts of the different enzymes were measured in subcellular fractions of myeloma cells, RNA polymerase I was found predominantly in the nucleolar fraction, II and III*, predominantly in the nucleoplasmic fraction, and IIIβ predominantly in the cytoplasmic fraction. This latter observation raises the possibility that this enzyme may be of cytoplasmic origin.

Cellular levels of the RNA polymerases have been compared in MOPC 315 tissues and in normal tissues under various physiological conditions. Levels of Enzymes I and III are increased in the myeloma relative to calf thymus, mouse liver, and mouse spleen, and are increased in liver and spleen from tumor-bearing mice and younger (6 weeks) mice relative to tumor-free mice and older mice (10 weeks), respectively. Levels of Enzyme II are more invariant under these circumstances. These data are discussed in terms of possible regulatory roles of the three enzyme classes.

The specific means by which eukaryotic cells achieve selective gene transcription are largely unknown; but a variety of regulatory mechanisms (both positive and negative) may be anticipated. It is also probable that knowledge of the structure, function, and regulatory features of the multiple cellular RNA polymerases (1) will be essential for understanding at least some aspects of transcriptional control. There are at least three classes of these enzymes which are designated I, II, and III (1, 2). Nucleolar RNA polymerase I (3) synthesizes rRNA (4-7); nucleolasmic RNA polymerase II (3) synthesizes RNA-like RNA1 (4-6) which presumably includes mRNA; and nucleoplasmic RNA polymerase III (3) transcribes the genes for 4 S RNA and 5 S RNA (7). Thus these enzymes together may account for most if not all nuclear RNA synthesis.

Consistent with the hypothesis that the enzymes perform distinct and essential functions, RNA polymerases I and II have

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been detected in virtually all cell types examined (1, 2). By contrast, RNA polymerase III has been detected in some (1, 8-13), but not all cell types (2, 14), possibly due to enzyme instability in certain cell types or simply to the use of inadequate detection methods. The levels of activity of Enzymes I, II (6, 8, 14-18), and III (8) in solubilized extracts vary for different cell types and for the same cell type under different physiological conditions, suggesting that the respective genes which the enzymes transcribe may be regulated in part via alterations in enzyme activity levels within the cell.

Structural studies have provided additional evidence for distinct functional roles of the RNA polymerases. Calf thymus RNA polymerases I and II have distinct subunit patterns (18, 19) while similar subunit patterns are found for RNA polymerase II from calf thymus (19, 20), rat liver (20), and human KB cells (21). Although RNA polymerase III from Xenopus laevis oocytes has a subunit structure distinct from that of RNA polymerases I and II, similar studies have not yet been reported for the mammalian Enzyme III. The general difficulty in obtaining sufficient amounts of all enzymes from many biological systems has hindered further analysis of enzyme structure-function relationships and has prohibited the use of homologous RNA polymerases for analysis of possible enzyme specific factors (9, 21-25) or chromosomal components (26, 27) in the regulation of gene transcription.

In order to investigate further the RNA polymerases and transcriptional controls in mammalian cells, the mouse myeloma system (28) has been chosen. Myeloma cells propagate rapidly (suggesting high enzyme levels) either in tissue culture or as solid or ascites tumors in BALB/c mice, and the use of these homogeneously cloned cell populations minimizes possible RNA polymerase variability due to heterotypic cell types. It is possible in this single cell type to study transcriptional controls related to a number of biological phenomena. The cells are highly malignant, and analysis of their RNA polymerases might reveal enzymatic differences characteristic of tumor cells in general. For example, enlarged nucleoli present in myeloma cells and typical of rapidly growing tumor cells (29) indicate high levels of RNA synthesis which may in turn reflect abnormally high levels of nucleolar RNA polymerase activity. Furthermore, induction, or maintenance, or both, of the differentiated state might be investigated since in most myeloma cells a substantial fraction of their translational and transcriptional capacities are geared toward the product, respectively, of a particular immunoglobulin and its mRNA (28). Finally, picorna virus pathology can be studied since myeloma cells can be superinfected with encephalomyocarditis virus which results in the expected inhibitions of host protein and RNA synthesis.

In this paper techniques are detailed for the detection, isolation, and characterization of the three classes of RNA polymerase from MOPC 315 tumors (30) as a basis for subsequent analysis of their structural, functional, and regulatory properties. In addition, cellular levels of solubilized RNA polymerase activities from various tissues in different physiological states are compared with cellular levels in MOPC 315 tumors.

**EXPERIMENTAL PROCEDURES**

**Cells**

MOPC 315 solid tumors were propagated subcutaneously in 6- to 10-week-old BALB/c mice and were kindly supplied by Doctors E. Simms and H. Eisen (Washington University School of Medicine). Tumors were dissected from mice 2 to 3 weeks after implantation and weighed 1 to 3 g each. Mouse livers and spleens were dissected from 6- or 10-week-old BALB/c mice which did not carry MOPC 315 tumors and from tumor-bearing mice. Calf thymus was obtained fresh from the slaughterhouse.

**Biochemicals**

Unlabeled nucleoside triphosphates were obtained from P-L Biochemicals. [H]UTP from New England Nuclear, crystalline bovine serum albumin from Pentex. Type I calf thymus DNA from Sigma, poly[d(A-T)] from P-L Biochemicals, and a-amanitin from Henley and Co.

**Preparation of Ion Exchange Resins**

CM-Sephadex (C-25) and DEAE-Sephadex (A-25) were purchased from Pharmacia. The resins were suspended in 2 to 3 volumes of water and adjusted to pH 7 with 1 M Tris-OH. The resins were washed with 20 volumes of 0.5 M ammonium sulfate (pH 7.9) followed by 10 volumes of water, and then equilibrated with 2 volumes of 0.05 M Tris-HCl (pH 7.9, 23°), 25% (v/v) glycerol, 0.01 M EDTA, 0.5 M ammonium sulfate, and 0.01% tolue. The suspensions were stored at 0-4° and prior to use were deaerated under vacuum.

**Conductivity, DNA, and Protein Measurements**

As previously reported (11) salt concentrations were measured with a Radiometer conductivity meter (type CDM-2) following 200-fold dilutions of the samples with water. DNA was measured according to the method of Burton (31). Protein was measured according to the method of Lowry et al. (32) after the samples were precipitated with trichloroacetic acid.

**Assay for RNA Polymerase**

Assays were performed in a final volume of 50 µl. Thirty-microliter aliquots of substrate solution were distributed initially and each contained 2.5 µmoles of Tris-HCl (pH 7.9), 0.1 µmole of MnCl₂, 0.03 µmole of GTP, CTP, and ATP, 0.0025 µmole of unlabeled UTP, 1 µCi of [H]UTP (10 to 20 Ci per mmole), 5 to 6 µg of native calf thymus DNA or 2.5 µg of poly[d(A-T)], and the appropriate amount of ammonium sulfate. Appropriate amounts of a-amanitin, when used, were included in the substrate solution. Reactions were initiated with the addition of 20-µl aliquots of the enzyme solutions. After 20 min at 37° reactions were terminated and [H]UMP-labeled RNA was measured as described (11). One unit of activity represents incorporation of 1 pmole of UMP into RNA in 20 min under the previously described conditions. For a given series of experiments the same DNA batch was always used because RNA polymerase activity varied among different template preparations.

**Cell Fractionation Procedures**

**Nuclei and Cytoplasm**—Nuclei from MOPC 315 cells were isolated by modification of procedures described previously (3, 33, 34). Tumors (100 g) were mixed with 300 ml of 0.04 M sucrose, 15 mM MgCl₂, and 0.25 mM spermidine, and homogenized (15 strokes) in a loose, hand-driven, glass-Teflon homogenizer. After filtration through two layers of cheesecloth, the homogenate was centrifuged at 2000 x g for 10 min. The supernatant fraction (cytoplasm) was removed, adjusted to 20% (v/v) glycerol, 0.05 M Tris-HCl (pH 7.9), and 0.5 mM diethytheritol, and either used immediately for solubilization of RNA polymerase or in some cases stored at -80° prior to solubilization. In some cases the lower reddish pellets (crude nuclei) were resuspended in Buffer A (0.06 M Tris-HCl (pH 7.9, 23°), 25% (v/v) glycerol, 0.1 M EDTA, 0.5 mM diethytheritol) and used for enzyme solubilization. Otherwise, for isolation of purified nuclei the crude nuclear pellet was resuspended in 100 ml of 0.34 M sucrose, 15 mM MgCl₂, and 0.35 mM spermidine, and rehomogenized as before (five strokes). This homogenate was again filtered through two layers of cheesecloth and then diluted with 400 ml of 2.3 M sucrose. Aliquots (30 ml) were placed in nitrocellulose centrifuge tubes and underlaid with 5 to 7 ml of 2.3 M sucrose containing either 15 mM

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1. R. G. Roeder, unpublished observations.
2. C. Lawrence and R. Thach, personal communication.
MgCl₂ (for enzyme solubilization directly from purified nuclei) or no MgCl₂ (for nuclear isolation). After centrifugation at 170,000 g (Beckman SW 27 rotor) for 1 hour the supernatant fluids and pellets were discarded and the white nuclear pellets were resuspended either in Buffer A (for direct enzyme solubilization from nuclei) or in 150 ml of 0.34 M sucrose (for preparation of nucleoli and nucleoplasm).

**Nuclear Fractionation**—Nucleolar and nucleoplasmic fractions were obtained by modification of procedures described by Busch (34) and Roeder and Busch (3). The purified nuclear suspension in 0.34 M sucrose (preceding section) was sonicated at 0° in 25-ml aliquots for periods of 15 s with a Branson model W185 Sonifier (microtip, power setting 4) until nearly all the nuclei were disrupted (about 30 s total sonication time). These suspensions were underlaid with equal volumes of 0.9 M sucrose and the nucleoli pelleted by centrifugation at 2500 X g for 20 min in a swinging bucket rotor of the IEC PR6 centrifuge. The upper phase contained the nucleoplasmic fraction and was removed, adjusted to 20% (v/v) glycerol, 0.05 M Tris-HCl (pH 7.9), and 0.5 mM dithioerythritol, and either subjected to the RNA polymerase solubilization procedure immediately or stored frozen at -80°.

The nucleolar pellets were resuspended in a small volume of 0.34 M sucrose and suspended microscopically pure. The nucleoli to nuclei ratio was always greater than 100. One volume of 2-fold concentrated Buffer A was added to the nucleolar suspension and RNA polymerase was solubilized as described below.

**RNA Polymerase Solubilization**—RNA polymerase was solubilized by similar procedures from whole cells, nuclei, cytoplasm, nucleoli, and nucleoplasm. Whole tissues (MOPC 315 tumors, calf thymus, mouse spleen, or mouse liver) were first homogenized in 0.34 M sucrose (preceding section) was sonicated at 0° in 25-ml volumes of Buffer A (3 ml per g of tissue). Subcellular fractions in Buffer A were obtained as described in the two preceding sections. Each preparation was adjusted to 0.32 M ammonium sulfate by the addition of 4 M ammonium sulfate (adjusted to pH 7.9 with ammonia). The viscous solutions were sonicated for 15 s periods with a Branson model W185 Sonifier (ultramicroprobe, power setting 4) until drops readily formed at the tip of a Pasteur pipette (about 1 min total sonication time). These suspensions were centrifuged for 50 min at 50,000 rpm in either a Spinco type Ti-60 or a type 50 rotor. The 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. The 0.1 M ammonium sulfate supernatant fractions (F2) were diluted to 0.05 to 0.07 M ammonium sulfate with Buffer A and centrifuged as before. These 0.05 to 0.07 M ammonium sulfate supernatant fractions (F3) were stored frozen at -80° where RNA polymerase activity was completely stable. RNA polymerase activities and protein contents were measured at F1, F2, and F3 as described. All procedures were performed at 0°.4

**Ion Exchange Chromatography**—Columns containing DEAE-Sephadex were equilibrated with Buffer A containing 0.05 M ammonium sulfate. Fractions of F3 (preceding section) were thawed and loaded at about 2 mg of protein per ml of DEAE-Sephadex bed volume. The columns were then washed with Buffer A containing 0.05 M ammonium sulfate and developed with a 2 to 3 column volume linear gradient of 0.05 to 0.5 M ammonium sulfate in Buffer A. Fractions equivalent to 3 to 5% of the total gradient volume were collected at a rate of about 1 ml of eluent per min for 20 ml of bed volume. Rechromatography of enzyme fractions (after CM-Sephadex chromatography, see below) on DEAE-Sephadex was performed in a similar fashion except that only 0.5 to 1.0 mg of protein was loaded per ml of bed volume.

In some cases MOPC 315 tumor RNA polymerase III fractions obtained from an initial DEAE-Sephadex chromatography were further fractionated by CM-Sephadex chromatography. The appropriate DEAE-Sephadex fractions were combined and dialyzed against 25 volumes of Buffer A containing 0.004 M ammonium sulfate until the ammonium sulfate concentration in the sample reached 0.06 M. A column containing CM-Sephadex was equilibrated with Buffer A containing 0.06 M ammonium sulfate. The enzyme was loaded at about 0.5 mg of protein per ml of CM-Sephadex bed volume. The column was washed with 1.5 column volumes of 0.05 M ammonium sulfate in Buffer A and the bound enzyme was then eluted with 0.15 M ammonium sulfate in Buffer A. Fractions equivalent to 10% of the bed volume were collected at a rate of 1 ml of eluent per min per 40 ml of bed volume.

**RESULTS**

**Purification and Chromatographic Properties**—The techniques employed for solubilization and chromatographic resolution of the RNA polymerases from MOPC 315 tumors are described under "Experimental Procedures." Relative to many other tissues these tumors contain high levels of RNA polymerase activity (see below) and the yields of enzyme activity obtained from 10 g of tumor (containing 53 mg of DNA) in a representative experiment are presented in Table I. The apparent enzyme activity continues to increase at each purification stage until DEAE-Sephadex chromatography, possibly due to removal of nonspecific inhibitory substances. DEAE-Sephadex chromatography of F3 yields four peaks of RNA polymerase activity as illustrated in Fig. 1. RNA polymerase I elutes at about 0.1 M ammonium sulfate and is insensitive to low concentrations (0.5 µg per ml) of α-amanitin. The two additional peaks of enzyme activity which are insensitive to 0.5 µg per ml of α-amanitin and which elute, respectively, at about 0.24 and 0.30 M ammonium sulfate in Fig. 1, are designated IIIA and IIIb. The salt concentrations at which IIIA and IIIb elute from DEAE-Sephadex have tended to vary somewhat with different gradient conditions. However, IIIA elutes either with or just after II while IIIb always elutes after IIIA. The rationale for designating the latter two activities as Class III enzymes is presented below. The cellular levels of these solubilized RNA polymerase activities are considered later.

As described previously for other systems (1) the major RNA polymerases, I and II, maintain their distinctive chromatographic properties when subjected to rechromatography on DEAE-Sephadex.4 This is also true for the two RNA polymerase III forms. For these experiments Enzymes IIIA and IIIB from DEAE-Sephadex fractions were further purified and separated from RNA polymerase II by chromatography on CM-Sephadex, which retains only RNA polymerase III (Fig. 2). RNA polymerase II elutes in the breakthrough fractions and is identified by its com-

**Table I**

<table>
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<th>Fraction</th>
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<th>Total activity</th>
<th>Specific activity</th>
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<tr>
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<td>1.600</td>
<td>96,000</td>
<td>0.06</td>
</tr>
<tr>
<td>F2</td>
<td>1.020</td>
<td>398,400</td>
<td>0.39</td>
</tr>
<tr>
<td>F3</td>
<td>700</td>
<td>421,600</td>
<td>0.53</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>205</td>
<td>204,400</td>
<td>1.29</td>
</tr>
<tr>
<td>II</td>
<td>80</td>
<td>126,600</td>
<td>1.58</td>
</tr>
<tr>
<td>IIIA</td>
<td>100</td>
<td>51,200</td>
<td>0.54</td>
</tr>
<tr>
<td>IIIB</td>
<td>32</td>
<td>26,900</td>
<td>0.84</td>
</tr>
</tbody>
</table>

4 L. B. Schwartz, unpublished observations.
FIG. 1. DEAE-Sephadex chromatography of RNA polymerases from MOPC 315 tumors. The RNA polymerase activity present in 10 g of MOPC 315 tumor was solubilized and Fraction F3 subjected to chromatography on a column (325 ml) of DEAE-Sephadex as described under “Experimental Procedures.” Fractions of 20 ml were collected and activity was measured at the salt concentration resulting from dilution of 20 μl of each column fraction to a final volume of 50 μl as described under “Experimental Procedures.” Measurements were performed with calf thymus DNA in the absence (— — — —) or presence (●——●) of 0.5 μg per ml of α-amanitin or with poly[d(A-T)] in the presence of 0.5 μg per ml of α-amanitin (▲——▲). , ammonium sulfate concentration; , absorbance at 260 nm.

Fig. 2. Resolution of RNA polymerases II and IIIα obtained from DEAE-Sephadex by CM-Sephadex chromatography. DEAE-Sephadex fractions containing both RNA polymerase II and IIIα activity were obtained from an experiment analogous to that shown in Fig. 1. A sample containing 14,640 units of IIIα activity (insensitive to 0.5 μg per ml of α-amanitin), 62,460 units of II activity (sensitive to 0.5 μg per ml of α-amanitin), and 10 mg of protein was chromatographed on a column (1.8 × 3.7 cm) of CM-Sephadex. Fraction volumes of 3.5 ml (Fractions 1 to 12) or 1.0 ml (Fractions 13 to 22) were collected. Recoveries of activity were 70% for II and 98% for IIIα. ●——●, activity in the presence of 0.5 μg per ml of α-amanitin; ○——○, activity minus α-amanitin; ———, ammonium sulfate concentration.

Properties of RNA Polymerases from MOPC 315 Tumors—In the following sections the Class I, II, and III enzymes are further distinguished on the basis of their differential sensitivities to α-amanitin, their ionic strength and divalent metal cation activation profiles, relative activities with DNA and poly[d(A-T)], and subcellular localizations.

α-Amanitin Sensitivity—α-Amanitin sensitivities were evaluated with native calf thymus DNA under standard assay conditions as shown in Fig. 4. RNA polymerase I is insensitive to α-amanitin concentrations of at least 400 μg per ml. RNA polymerase II is inhibited by 50% at 0.025 μg per ml of α-amanitin and completely inhibited at 0.5 μg per ml. Although RNA polymerases IIIα and IIIβ are both inhibited by α-amanitin, their inhibition curves (which are essentially superimposable) are clearly displaced from the II inhibition curve. IIIα and IIIβ are virtually uninhibited at 0.5 μg per ml of α-amanitin but show complete inhibition at 400 μg per ml with their 50% inhibition points at about 20 μg per ml of α-amanitin. The same results have been obtained for IIIα and IIIβ using poly(dA-T) as templates. Similar results were previously found for RNA polymerase III from X. laevis oocytes and somatic cells.

R. Weinmann, unpublished observations.
Ammonium Sulfate Activation Profiles—RNA polymerase activities were also measured as a function of ammonium sulfate concentration as illustrated in Fig. 5. With calf thymus DNA, RNA polymerases I and II have optima, respectively, at 0.055 and 0.09 m ammonium sulfate, similar to values reported for the analogous enzymes from other tissues (1, 2). As previously reported for Enzyme III from X. laevis (11), RNA polymerases IIIA and IIIB from MOPC 315 tumors show biphasic profiles with maxima at 0.055 and 0.17 m ammonium sulfate. II, however, the ionic strength activation profiles with poly[d(A-T)] as template and in the presence and absence of 0.5 μg per ml of α-amanitin. RNA polymerase II activity (not shown) was detected by its sensitivity to α-amanitin and the elution position of this enzyme is indicated by an arrow in each experiment. activity resistant to 0.5 μg per ml of α-amanitin; −−−, ammonium sulfate concentration.

Divalent Cation Activation Profiles—The activation profiles of MOPC 315 RNA polymerases I and II by Mn2+ and Mg2+ are similar to those reported for enzymes from other tissues (1) as illustrated in Fig. 6. RNA polymerase I activity is about the same whether optimal concentrations of Mn2+ or Mg2+ are used, while polymerase II activity is about 3-fold greater with Mn2+ than with Mg2+ at optimal concentrations of each ion. For RNA polymerases IIIA and IIIB at optimal metal ion concentrations the Mn2+:Mg2+ activity ratios are identical at 0.05 m and at 0.17 m ammonium sulfate. Each enzyme shows about 2-fold greater activity with Mn2+. Similar observations were made for RNA polymerase III from sea urchin embryos (1) and from X. laevis cells (11).
FIG. 5. Effect of ammonium sulfate upon the activity of the RNA polymerases. Samples of RNA polymerases I, II, IIIA, and IIIB were obtained as indicated in Fig. 4 and were dialyzed against Buffer A until the ammonium sulfate concentrations reached 0.93 to 0.96 M. Aliquots were then assayed at the indicated salt concentrations with calf thymus DNA (O--O) or with poly[d(A-T)] (O---O) as described under “Experimental Procedures.” A, RNA polymerase I; B, RNA polymerase II; C, RNA polymerase IIIA; D, RNA polymerase IIIB. With calf thymus DNA the maximal activity values were 6,530 units per ml (I); 2,100 units per ml (II); 5,460 units per ml (IIIA); and 9,511 units per ml (IIIB). With poly[d(A-T)] the corresponding values were 7,699 units per ml (I); 1,269 units per ml (II); 12,900 units per ml (IIIA); and 18,571 units per ml (IIIB).

Those made previously for rat liver nuclear RNA polymerases (3). The small amount of IIIB detected in the crude nuclear fraction (Table II) suggests that this activity may be lost during subsequent purification of nuclei (Fig. 7) although contributions of IIIB due to cytoplasmic contamination cannot be ruled out.

Exact quantitation is difficult in these studies because the milieu to which the enzymes are exposed in each subcellular fraction is variable. Thus the fractionation procedures probably result in some variations in enzyme stabilities as well as in their apparent activities (due to different populations of contaminating proteins). This is the most likely explanation for the lack of coincidence, in some cases, between activity in a given fraction and the combined activities of the derived fractions. In the case of RNA polymerase I, for example, the measured activity in the isolated nucleoli routinely is found to be greater than that measured in purified nuclei, probably as a result of being highly concentrated and relatively pure in the former case. Nonetheless, these problems in quantitation do not detract from the validity of the overall generalizations concerning the primary subcellular localizations of the enzymes after cellular fractionation.

Another relevant observation with respect to the actual in vivo localizations of the enzymes is that nuclei isolated at low concentrations of Mg2+ contain less of the total cellular amounts of RNA polymerases II and IIIA than do the more intact nuclei isolated at higher Mg2+ concentrations, presumably a result of nuclear leakage. The activity lost from nuclei at low Mg2+ concentrations is recovered in the cytoplasmic fractions. Because of these observations and of the previous demonstrations that RNA polymerases may leak from nuclei isolated by aqueous techniques (10, 36, 37), attempts were made to increase retention of RNA polymerase IIIB. Divalent cations such as Mn2+, Ca2+, and Mg2+ were employed alone or in combination in the nuclear isolation media; but still no IIIB could be detected in such nuclei. Thus the question of whether or not IIIB functions or exists in the nucleus remains unanswered.

RNA Polymerase Levels in Different Tissues—Levels of the RNA polymerases have also been measured in calf thymus and in BALB/c liver and spleen tissue taken from 10-week-old mice. Normal, healthy mice and mice in which tumors had been growing for 3 weeks were analyzed. RNA polymerase was solubilized by standard procedures. No losses in activity were apparent in Fractions F1, F2, and F3. Fractions F3 were then analyzed via DEAE-Sephadex chromatography as illustrated in Fig. 8, A to E. Class I, II, and III RNA polymerases are clearly present in each tissue. Forms IIIA and IIIB are both present in MOPC 315 tumors and in liver and spleen tissue from BALB/c mice carrying MOPC 315 tumor. In normal BALB/c liver IIIB predominates; in normal BALB/c spleen IIIA appears to predominate. In calf thymus, low levels of III activity are detected with poly[d(A-T)] as template. Whether or not this Class III activity represents IIIA or IIIB remains to be seen. RNA polymerase activity levels have been calculated for I, II, and III (IIIA + IIIB) from the DEAE-Sephadex results and normalized to DNA in order to facilitate comparisons of relative cellular levels (Table III). The cellular levels of MOPC 315 RNA polymerases appear significantly higher than those in other normal tissues. Thus, the level of RNA polymerase I is 2- to 20-fold higher, that of II 1.5- to 2.5-fold higher, and that of III 2.5- to 25-fold higher. In addition, the spleen and liver tissues of BALB/c mice carrying MOPC 315 tumors contain significantly higher cellular levels of RNA polymerases I and III activity than do the same tissues from normal mice. By contrast the cellular
FIG. 6. Effects of Mg$^{2+}$ and Mn$^{2+}$ upon the activities of the RNA polymerases. RNA polymerases I, II, III$\alpha$, and III$\beta$ were obtained as described in Fig. 4 and were assayed at the indicated metal ion concentrations as described under "Experimental Procedures." A, RNA polymerase I; B, RNA polymerase II; C, RNA polymerase III$\alpha$; D, RNA polymerase III$\beta$. •—•, activity in the presence of Mg$^{2+}$ and 0.05 mM ammonium sulfate; O-O, activity in the presence of Mn$^{2+}$ and 0.05 mM ammonium sulfate; •—•, activity in the presence of Mg$^{2+}$ and 0.17 M ammonium sulfate; O—O, activity in the presence of Mn$^{2+}$ and 0.17 M ammonium sulfate.

RNA polymerase II levels are relatively invariant in these tissues under different physiological conditions.

DISCUSSION

Following solubilization of RNA polymerase activity from MOPC 315 cells, four forms of the enzyme were separated by ion exchange chromatography. The first major peak of activity eluting from DEAE-Sephadex corresponds to the α-amanitin-insensitive RNA polymerase I, while the second major peak of activity corresponds to RNA polymerase II as verified by its extreme sensitivity to α-amanitin (50% inhibition at 0.025 µg per ml). These two enzymes appear similar to the analogous enzymes in other systems with respect to their chromatographic properties, α-amanitin sensitivities, divalent cation, and ammonium sulfate activation profiles (with calf thymus DNA), and subcellular localizations (below). In other systems, heterogeneity has been shown in RNA polymerase I (reviewed in Refs. 11 and 35) and in RNA polymerase II (11,19,20). The question of MOPC 315 RNA polymerase I heterogeneity is considered in the accompanying paper (35). Preliminary structural analyses of the MOPC 315 RNA polymerase II suggest a heterogeneity similar to that described in other systems (19,20). However, in the cases examined (11) the respective heterogeneous forms appear to have identical catalytic properties. Hence these enzymes are designated Class I and Class II RNA polymerases.

Two minor forms of RNA polymerase activity are categorized as Class III enzymes (III$\alpha$ and III$\beta$) because they elute from DEAE-Sephadex at high ionic strengths and because they are insensitive to low concentrations (0.5 µg per ml) of α-amanitin as previously reported for other Class III enzymes (1,10–12). However, the present data show that higher concentrations of α-amanitin completely inhibit this activity. Both RNA polymerases III$\alpha$ and III$\beta$ are equally sensitive although the α-amanitin concentration required for 50% inhibition is about 1000 fold greater than that required to inhibit RNA polymerase II to the same extent. This represents a specific effect since RNA polymerase I is totally unaffected by high toxin concentrations. The differential sensitivities of RNA polymerases I, II, and III thus make it possible to ascertain the relative proportions of each RNA polymerase class in unfractionated systems. It seems unlikely that an impurity in the α-amanitin samples inhibits RNA polymerase III since three different preparations were found to be equally effective in inhibiting this enzyme.

Other catalytic and chromatographic properties common to Enzymes III$\alpha$ and III$\beta$ clearly distinguish them from the Class I and Class II enzymes. The poly[d(A-T)] to native DNA activity ratio is greatest for the Class III enzymes. Furthermore, the ammonium sulfate activation profiles of RNA polymerases III$\alpha$ and III$\beta$ with native DNA template are biphasic, whereas with a poly[d(A-T)] template the high salt activation is abolished and a monophasic pattern results. By contrast the monophasic ammonium sulfate profiles for Enzymes I and II are not appreciably altered by the change from native DNA to poly[d(A-T)] as template. The possible biological significance of the selective ability

FIG. 7. DEAE-Sephadex chromatography of RNA polymerases from purified nuclear and from cytoplasmic fractions. Subcellular fractions were obtained and the RNA polymerase activity in those fractions solubilized and chromatographed on DEAE-Sephadex as described under "Experimental Procedures." In A, a cytoplasmic enzyme preparation (Fraction F3) from 5.6 g of myeloma tissue was chromatographed on a column (60 ml) of DEAE-Sephadex and 4-ml fractions were collected. In B, a nuclear enzyme preparation (Fraction F3) corresponding to 12 g of tissue (estimated from the DNA content as described in Table II) was chromatographed on a 50-ml column and 4.5-ml fractions were collected. RNA polymerase activity was measured with calf thymus DNA in the absence of a-amanitin and in the presence of 0.5 µg per ml of a-amanitin; and with poly[d(A-T)] in the presence of 0.5 µg per ml of α-amanitin (A—•—•—•—•), ammonium sulfate concentration.
of the Class III enzymes to initiate and transcribe natural template at high ionic strengths is currently unknown.

RNA polymerases IIIa and IIIb can be separated completely from Enzyme I (by DEAE-Sephadex chromatography) and from Enzyme II (by CM-Sephadex chromatography). On phosphocellulose IIIa and IIIb elute prior to RNA polymerase I, but unexpectedly they elute coincidentally with RNA polymerase I upon DEAE-cellulose chromatography. Similar results were previously found with RNA polymerases I and III from X. laevis oocytes and embryos. Because RNA polymerase III activity can be masked easily by the higher activity of RNA polymerase I in gradient fractions from DEAE-cellulose (or CM-Sephadex), it may have previously escaped detection in some eukaryotic systems, e.g. calf thymus (14). Using the more sensitive detection methods described in this report, RNA polymerase III activity has been detected in all cell types thus far examined, including calf thymus.

Subcellular Localization and Function of RNA Polymerases—As reported for other eukaryotic systems (3), RNA polymerases I and II from MOPC 315 tumors were detected primarily in nucleolar and nucleoplasmic fractions (3), respectively. These findings are consistent with the function of RNA polymerases I and II in the synthesis, respectively, of rRNA in the nucleolus (4–7) and DNA-like RNA in the nucleoplasm (4–6). RNA polymerase III has been detected previously in nuclei (1, 4, 8, 12) and further localized in nucleoplasmic fractions (3), as is the case for RNA polymerase IIIA from MOPC 315 tumors. By contrast, RNA polymerase IIIb has been found in cytoplasmic and crude nuclear fractions, but not in purified nuclei and, hence, could be of cytoplasmic origin. The possibility of nuclear leakage, however, has not been ruled out (see Ref. 38). A putative cytoplasmic RNA polymerase has been reported in rat liver (39) and resembles RNA polymerase III from X. laevis oocytes and somatic cells and from myeloma cells with respect to α-amanitin sensitivity and certain chromatographic properties and may in fact be analogous to MOPC 315 RNA polymerase IIIb. Recently a Class III RNA polymerase activity has been implicated in 4S and 5S RNA synthesis (7) in isolated MOPC 315 nuclei. However, because two Class III enzymes with potentially the same location (in the nucleus) are detected, it is not yet possible to assign distinct functions to either IIIA or IIIb.

Regulation of RNA Polymerase Activities—MOPC 315 tumors contain higher cellular levels of solubilized RNA polymerase activity than do normal mouse spleen and liver or calf thymus tissues. Presumably this reflects the rapid growth rate, or the high levels of protein synthesis characteristic of these malignant tumors, or both. The cellular activity levels of RNA polymerases I and III also fluctuate considerably from tissue to tissue while those of RNA polymerase II are more invariant. Variations in physiological conditions also appear to influence cellular levels of RNA polymerase activity within the same tis-
Levels of RNA polymerase activities in different tissues.

Data were calculated from the DEAE-Sephadex chromatographic analyses of solubilized enzyme preparations shown in Fig. 1 and in Fig. 8. The DNA levels per g of tissue were determined as described under “Experimental Procedures” and the enzyme activity expressed as units per mg of DNA. RNA polymerase III activity represents the sum of III A and III B activities measured with calf thymus DNA as template and in the presence of 0.5 μg per ml of α-amanitin (data not shown in Fig. 8). Values for MOPC 315 tumors represent the average of five preparations while those for BALB/c livers and spleens from normal or tumor-bearing mice and those for calf thymus represent the average of two experiments.

<table>
<thead>
<tr>
<th>Tissue fraction</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPC 315 tumors</td>
<td>5500</td>
<td>2500</td>
<td>1100</td>
</tr>
<tr>
<td>BALB/c liver</td>
<td>764</td>
<td>1350</td>
<td>185</td>
</tr>
<tr>
<td>MOPC 315 BALB/c liver</td>
<td>2380</td>
<td>1780</td>
<td>292</td>
</tr>
<tr>
<td>BALB/c spleen</td>
<td>210</td>
<td>1138</td>
<td>137</td>
</tr>
<tr>
<td>MOPC 315 BALB/c spleen</td>
<td>1150</td>
<td>1380</td>
<td>310</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>527</td>
<td>1040</td>
<td>46</td>
</tr>
</tbody>
</table>

The levels of RNA polymerases I and III in spleen and liver from mice carrying MOPC 315 tumors increase several-fold relative to levels in tissues from healthy mice of the same age, while increases of RNA polymerase II levels are far less dramatic. This may result from general effects of the malignant tumor on the whole organism or, in the case of the affected spleen from the tumor-bearing mouse (which is considerably enlarged), may reflect the increased growth rates of these younger tissues.

These modulations of cellular RNA polymerase activities suggest regulation at the level of RNA polymerase (or polymerases). Whether such modulations are mediated via changes in the concentrations of the enzymes or via alterations (e.g. by effector molecules) in the activity of the enzymes is not known. It is clear, however, that the enzymes which show the greatest variations in cellular activity levels are those which appear to transcribe predominantly the ribosomal RNA genes (RNA polymerase I) and the 4 S and 5 S RNA genes (RNA polymerase III A or III B, or both), all of which are highly redundant and clustered in the eukaryotic cell genome (40–49). These observations suggest that the activities of the respective genes may be regulated by similar mechanisms operative at the level of the enzymes.

By contrast the levels of RNA polymerase II are more invariant, suggesting either invariant cellular requirements for total DNA-like RNA synthesis or normal cellular excesses of this enzyme. Furthermore, the suggestion that RNA polymerase II transcribes an extremely heterogeneous group of genes (4–6) implies that the levels of this enzyme alone cannot differentially regulate the rates of synthesis of specific gene products and that additional components may direct enzyme specificity, or activity in vivo, or both.

Purification and subunit structure of nucleolar RNA polymerase I has been examined in the accompanying paper (55).

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Isolation and Partial Characterization of the Multiple Forms of Deoxyribonucleic Acid-dependent Ribonucleic Acid Polymerase in the Mouse Myeloma, MOPC 315
Lawrence B. Schwartz, Virgil E. F. Sklar, Judith A. Jaehning, Roberto Weinmann and Robert G. Roeder


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