Murine Myeloma Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerases. Enzyme Isolation and Characterization of Interactions with Native and Denatured Deoxyribonucleic Acid†

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ABSTRACT: The solubilization and fractionation of the DNA dependent RNA polymerases from murine myeloma (MOPC 21) are described. Two major species of activity are eluted from DEAE-Sephadex; the first (polymerase A) prefers a native DNA template and is insensitive to α-amanitin whereas the second, polymerase B, is sensitive to α-amanitin (95% inhibition) and demonstrates maximal activity on denatured DNA. Both enzymes are maximally stimulated by $(NH_4)_2SO_4$ at a concentration of 62 mM in the presence of Mn^{2+} at a concentration of 3 and 2 mM for polymerase A and B, respectively. The transcription of several homopolymeric polydeoxynucleotide templates revealed that of the single-stranded templates only poly(dC) supported significant incorporation of the complementary nucleotide. The double-stranded template poly(dC)·poly(dI) was asymmetrically transcribed into poly(rG) and supported 1.7 and 13.7 times the incorporation of rG with polymerase A and B, respectively, relative to native myeloma DNA. Polymerase A transcribed poly(dA-T)·poly(dA-T) at a rate comparable to native DNA whereas polymerase B incorporated three times the ribonucleoside monophosphate, relative to native DNA. Using salmon sperm DNA, polymerase A demonstrated a fivefold preference for native over the denatured conformation while polymerase B preferred the denatured template. Both enzymes exhibit a higher affinity for denatured DNA and A appears incapable of rapid chain elongation on this template. With homologous myeloma DNA polymerase A catalyzes relatively short RNA chains (4–5S) on either native or denatured DNA and undergoes continuous reinitiation *in vitro*. Polymerase B remains firmly bound to native myeloma DNA through an initial 10 min and thereafter undergoes reinitiation. Transcription of native myeloma DNA *in vitro* by polymerase B yields a larger product with a bimodal distribution (6–7S and 10–11S). The 6–7S component is absent in the profile of RNA synthesized on denatured DNA by polymerase B.

Multiple mammalian DNA dependent RNA polymerases[1] exhibit different template specificities for natural deoxyribonucleic acids (DNAs) (Gniazdowski *et al.*, 1970), and synthetic polydeoxynucleotides (Blatti *et al.*, 1970), suggesting that each enzyme is capable of discriminating between different template sequences. These enzymes also initiate chain formation at different sites (Meilhac and Chambon, 1973) and synthesize product RNAs of different nearest neighbor frequency from a homologous DNA template (Smuckler and Tata, 1972) supporting this contention. The selection of specific sites for transcription points to a homology with the bacterial enzyme, suggesting that regulation of gene expression in mammalian cells could be accomplished, in part, by the relative abundance of polymerases of differing transcriptional specificities.

Mandel and Chambon (1974a,b) have concluded that the purified mammalian enzymes resemble *Escherichia coli* core polymerase lacking some essential factor necessary for specific initiation on intact DNA. Several protein "factors" have been prepared from mammalian cells and are purported to interact specifically with the polymerases resulting in an increase in their rate of transcription of native DNA (Siefert, 1970a; Stein and Hausen, 1970a; Lee and Dahmus, 1973; Sugden and Keller, 1973), but there are no data to indicate that any of these "factors" play any σ-like role in regulating the locus specificity of binding and initiation as for the prokaryote enzyme (Hinkle and Chamberlin, 1972). A further complication of transcriptional regulation in the mammalian cell is that the template *in vivo* is in part covered with nucleoproteins, some of which may function in dictating the "available" regions (Marushige and Bonner, 1966; Paul and Gilmour, 1966; Tan and Miyagi, 1970; Spelsberg *et al.*, 1971; Cedar and Felsenfeld, 1973). Any exhaustive study of the template–enzyme interactions must inevitably include a careful investigation of the contribution that the various constituents of chromatin make to the transcriptional process.

As part of an attempt to identify the mechanisms involved in the regulation of the template interactions of mammalian RNA polymerases, the murine myeloma system was selected as a model. The advantages derived from these cells include: (1) their propagation in the intact animal and in tissue culture; (2) formation of a specific class of mRNAs that can be isolated and purified (myeloma light chain mRNA) (Schechter, 1973); and (3) the opportunity to critically assess the effects of available chemotherapeutic agents on the macromolecular synthesis of the cancer cell. The first phase of this study entailed the isolation of RNA polymerases from a strain of murine myeloma

† From the Department of Pathology, University of Washington, Seattle, Washington 98195. *Received September 19, 1973.* Supported in part by USPHS Grants CA-13600 and GM-13543, and American Cancer Society Grant NP-99C. S. H. H. is a Postdoctoral Trainee in experimental pathology supported by USPHS Grant GM-00100.

[1] Abbreviations used are: DNA dependent RNA polymerase, deoxyribonucleic acid-dependent ribonucleic acid polymerase, nucleosidetriphosphate:RNAnucleotidyltransferase (EC 2.7.7.6); SDS, sodium dodecyl sulfate; DTT, dithiothreitol; n-DNA, native DNA; d-DNA, denatured DNA; SSC, standard saline citrate, 0.15 M NaCl–0.015 M sodium citrate. $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; SV40 form I DNA, simian virus 40 closed-circular superhelical DNA.

(MOPC 21) and the characterization of their interactions with several natural and homopolymeric DNA templates.

Materials and Methods

Buffers. STKM buffer contained 0.05 M Tris-HCl (pH 7.4 at 20°), 0.025 M KCl, 0.005 M $MgCl_2$, 0.005 M β-mercaptoethanol, 0.25 M sucrose, and $PhCH_2SO_2F$ (0.05 mg/ml). STMD buffer contained 0.05 M Tris-HCl (pH 7.4 at 20°C), 0.025 M $MgCl_2$, 0.005 M DTT, 1.0 M sucrose, and 0.05 mg/ml of $PhCH_2SO_2F$. TGMED buffer contained 0.05 M Tris-HCl (pH 7.9 at 20°), 25% glycerol (v/v), 0.005 M $MgCl_2$, 0.1 mM Na_2EDTA, 0.5 mM DTT, and 0.05 mg/ml of $PhCH_2SO_2F$. To this buffer $(NH_4)_2SO_4$ was added to bring the final molarity to either 0.025 M (TGMED + 0.025 M $(NH_4)_2SO_4$) or 0.5 M (TGMED + 0.5 M $(NH_4)_2SO_4$). TGED buffer contained 0.01 Tris-HCl (pH 7.9), 20% glycerol, 0.001 M EDTA, 0.001 M DDT, and 0.05 mg/ml of $PhCH_2SO_2F$. Both DTT and $PhCH_2SO_2F$ were added to all buffers just prior to use.

Tumor Tissue. Murine myeloma, MOPC 21 or P3K (a clone of MOPC 21), was maintained in Balb/c mice by serial transplantation. Tumor fragments were placed in bilateral fascial pouches on the rear flank and routinely attained a weight of 4–5 g within 3 weeks. The animals were sacrificed by cervical dislocation; the tumor was dissected free of investing tissues and necrotic debris and minced in STKM buffer of 0–4°. Routine preparations employed 60–110 g of tumor. All further procedures were performed between 0 and 4° unless specified otherwise.

Isolation of Nuclei. Freshly excised tissue was placed in tared beakers containing 4 volumes (4 ml/g of tissue) of STKM buffer, minced, and then homogenized with 10 strokes of a Teflon-pestled device. The material was filtered through 100 mesh nylon bolting cloth and further homogenized in a Dounce homogenizer with six strokes with the tight-fitting pestle. This brei was centrifuged at 755*g* for 10 min and the resulting pellets were resuspended in one volume of STKM. An equal volume of STKM buffer containing 1.0% Nonidet P-40 was added, the suspension was gently dispersed with two strokes of a loose-fitting pestle of the Dounce homogenizer, and the nuclei were pelleted by centrifugation at 755*g* for 10 min. The nuclei were resuspended and washed twice.

Solubilization of DNA Dependent RNA Polymerase. RNA polymerase was solubilized and fractionated by a modification of the general procedure described by Roeder and Rutter (1970). Nuclei from approximately 100 g of tissue were suspended in STMD (2.5 ml/g of original tissue, final volume 250 ml) with 25 ml (0.1 ml/ml of suspension) of 4.0 M $(NH_4)_2SO_4$ (pH 7.9). The suspension was transferred to a 250-ml Branson rosette cell and sonicated with a Branson sonifier Model W185 using a 0.5 in. diameter horn at maximum power setting. Sonication was done in 30-sec bursts for a total of 10 min. The sonicated suspension was then mixed with 2 volumes of TGMED + 0.15 M $(NH_4)_2SO_4$ and centrifuged in a Beckman Spinco 50.1 rotor at 105,000*g* (max) for 1 hr. The supernatant was collected and solid $(NH_4)_2SO_4$ (0.42 g/ml) was added with stirring over a 30-min period and then stirred an additional 30 min at 0°. This suspension was centrifuged at 105,000*g* for 90 min and the resulting pellets were resuspended in TGMED plus 0.25 M $(NH_4)_2SO_4$ (0.5 ml/g of tissue).

Agarose Column Chromatography. Agarose (Bio-Gel A 1.5m) was equilibrated in TGMED plus 0.025 M $(NH_4)_2SO_4$, packed in a 2.5 × 100 cm column, and washed with three column volumes (1500 ml) of buffer. The sample was applied and the column was developed with the equilibrating buffer. The eluent was collected at 150 drops (approximately 4.1 ml)/tube and 0.05-ml aliquots were assayed as described under RNA Polymerase Assay. Fractions containing RNA polymerase activity were pooled and immediately applied to a DEAE-Sephadex column or frozen in liquid nitrogen.

DEAE-Sephadex Chromatography. DEAE-Sephadex (A25) was precycled, and then equilibrated with 0.025 M $(NH_4)_2SO_4$ in TGMED. The gel was poured into a 2.0 × 30 cm column, packed in one pass, and washed with 0.025 M $(NH_4)_2SO_4$ in TGMED until the conductivity of the eluent was identical with the original buffer.

The pooled RNA polymerase activity from the A 1.5m chromatography was adsorbed to the DEAE-Sephadex at a flow rate of 0.5 ml/min and the flow-through was collected at 4 ml/tube. The column was washed with one column volume of 0.025 M $(NH_4)_2SO_4$ in TGMED and the protein was eluted with a linear gradient of 0.025–0.5 M $(NH_4)_2SO_4$ in TGMED (65 × 65 ml). The eluent was collected in fractions of 1.7 ml/tube and two 0.03-ml aliquots were removed and assayed with either native or denatured DNA as described in Methods. Protein concentration and conductivities were determined for each column fraction. Protein concentrations were determined by the method of Bramhall (Bramhall *et al.*, 1969) or Lowry (Lowry *et al.*, 1951) using crystalline bovine albumin as a standard. Conductance was measured using a Radiometer conductance meter equipped with a micro conductance cell. The RNA polymerase activity was pooled.

Poly(rA)-Sepharose Chromatography of Enzyme A. Polyadenylate-Sepharose (poly(rA)-Sepharose) was synthesized according to Poonian *et al.* (1971). Following successive washes with 1.0 M aminoethanol in 0.05 M potassium phosphate buffer at pH 8.0, and 0.5 M potassium phosphate at pH 8.0, the gel was equilibrated with TGED buffer. The column (75 ml bed volume) was poured in one pass and washed with 500 ml of the suspending buffer. The DEAE-Sephadex pool of polymerase A was desalted on Sephadex G-25 and applied to the poly(rA)-Sepharose column at 0.5 column volume/hr. The column was washed with one column volume of buffer and eluted with a linear gradient of three column volumes from 0 to 0.5 M NaCl in TGED buffer.

Glycerol–Sucrose Gradient Centrifugation of Enzymes. Pools of polymerase A following poly(rA)-Sepharose chromatography and polymerase B after DEAE-Sephadex were precipitated with 0.42 g/ml of $(NH_4)_2SO_4$ and resuspended in TGED buffer modified to contain 0.05 M Tris-HCl (pH 7.9), 15% glycerol, and 0.05 M NaCl. The resuspended samples were dialyzed for 6 hr, layered on glycerol–sucrose gradients, and centrifuged as described by Sugden and Keller (1973). The gradients for polymerase A contained 0.025 mg/ml of $PhCH_2SO_2F$.

Phosphocellulose Chromatography of Polymerase A. Whatman P11 cellulose phosphate was precycled and equilibrated with 1.0 M $(NH_4)_2SO_4$ (pH 7.9 at 20°). After 30 min the slurry was decanted and equilibrated with TGED buffer containing 30% glycerol (v/v) and 0.05 M $(NH_4)_2SO_4$. Eluent fractions from DEAE-Sephadex chromatography containing polymerase A activity were dialyzed overnight against TGED + 0.05 M $(NH_4)_2SO_4$ and applied to the column at a flow rate of 1.0 ml/min. The column was washed with 20 ml of TGED + 0.05 M $(NH_4)_2SO_4$ and the polymerase activity eluted with a linear gradient of 0.05–0.5 M ammonium sulfate in TGED. Fractions were assayed as described using native salmon sperm DNA.

Assay of DNA Dependent RNA Polymerase. The standard assay (rate-limiting UTP) contained in a total volume of 0.08 ml: 0.7 mM ATP, GTP, and CTP; 6.25 μM [^{3}H]UTP (1 Ci/

mmol); 50 mM β-mercaptoethanol; 3.1 mM $MnCl_2$; 60 mM Tris-HCl (pH 7.9 at 20°); 25 μg of native or heat-denatured salmon sperm DNA; 60 mM $(NH_4)_2SO_4$ (except for DEAE-Sephadex assays); 10% glycerol (v/v); and 30 μl of enzyme suspended in TGMED buffer. Incubations were conducted at 37° for 30 min.

Assay conditions for the synthetic polydeoxynucleotide template experiments (nonlimiting UTP) were modified as follows. The reactions contained in 0.08 ml: all ribonucleoside triphosphates ([^{3}H]labeled and nonlabeled) at 1.0 mM each; synthetic polydeoxynucleotides added in increasing concentrations to attain saturating levels; 20 μl of enzyme in TGMED buffer; and other reagents as described in the standard assay. The double-stranded polydeoxynucleotide templates were synthesized by mixing an equal number of A_{260} units of each complementary single-stranded polydeoxynucleotide suspended in 1 × SSC. Following mixing, the samples to be annealed were heated to 95° for 30 sec, cooled to 65° and incubated at that temperature for 30 min, 50° for 30 min, and finally cooled to 20° (room temperature) over a 2–3-hr period. Glassware used with templates containing poly(dG), -(dC), or -(dI) was siliconized to minimize adsorption to glass. All glassware was acid-washed and autoclaved to minimize adventitious RNase contamination. The incubations were carried out at 37° for 10 min.

Following incubation, the tubes were placed on ice and 0.05-ml aliquots were removed and applied to 2.4 cm Whatman No. 1 filter paper discs, which were placed in ice-cold 10% Cl_3CCOOH containing 1.0% sodium pyrophosphate and processed as described (Saunders *et al.*, 1972). The dried discs were counted in 10 ml of toluene containing 0.1% 2,5-diphenyloxazole and 0.05% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in a Model 3375 Packard TriCarb liquid scintillation spectrometer. The counting efficiency for tritium using the filter paper disc method is 4.5%.

Assays for Ribonuclease and Deoxyribonuclease. Ribonuclease assays contained in 0.08 ml: 6500 cpm of [^{14}C]RNA labeled *in vivo* with [^{14}C]orotate and isolated from rat liver as described (Church and McCarthy, 1970); either 3.1 mM $MgCl_2$ or $MnCl_2$; either 90.0 or 185 mM $(NH_4)_2SO_4$; 40 μl of enzyme in TGMED; and other reagents as described for the standard assay excluding the ribonucleoside triphosphates and DNA. The reactions were stopped at appropriate time points by adding ¼ volume of a 1:1 mix of 5 mg/ml of yeast RNA and 4 M NaCl. Two volumes of ethanol at −20° were added and the samples kept in ice an additional 10 min, then centrifuged at 2000*g* for 20 min; the supernatants were aspirated and added to 1 ml of Protosol Tissue Solubilizer (New England Nuclear). These were incubated at 60° for 1 hr, and the total sample was added to 10 ml of toluene-based scintillation fluid for the radioactivity measurement.

Ribonuclease H assays were conducted by a modification of the method of Wyers *et al.*, (1973) using poly(dG)·poly([^{3}H]C) and poly(dT)·poly([^{3}H]rA) as substrates. Hybrids were prepared by mixing 3.0 A_{260} units of the polydeoxynucleotide with 1 μCi of the ^{3}H-labeled complementary polyribonucleotide in a total volume of 0.5 ml of 1 × SSC. The mixes were heated to 100° for 2 min and then incubated at 37° for 4 hr. Time points were taken throughout the incubation and the degree of resistance to 20 μg/ml of RNase A in 2 × SSC was monitored. The samples were digested with 20 μg/ml of RNase A for 15 min, passed over Sephadex G-50 equilibrated in 1 × SSC, and stored at −20°. Prior to use they were incubated at 37° for 15 min to assure a double-stranded conformation. The degree of degradation was assayed by spotting a 0.050-ml aliquot of control or RNase-treated hybrids onto 2.4-cm diameter Whatman No. 1 discs which were processed as described. Endodeoxyribonuclease activity was ascertained by the degree of conversion of closed circular polyoma DNA (component I) to nicked (component II) DNA. The assays were conducted in the standard RNA polymerase mix modified by using unlabeled UTP (1.0 mM) and [^{3}H]polyoma DNA form I (closed circular form). Incubations were performed at 37° and were stopped by pipetting 0.08-ml samples into an equal volume of ice-cold Tris-EDTA solution (0.01 M Tris-HCl (pH 8.0)–0.04 M EDTA). An equal volume (0.16 ml) of 0.3 M Na_3PO_4 was then added immediately and the samples were allowed to sit at room temperature for 5 min; 5 ml of 6 × SSC and 0.2 ml of 1.0 M Tris-HCl (pH 8.0) were added and mixed vigorously; the samples were then filtered through membrane filters (Schleicher and Schuell, Type B-6, 24 mm), which had been soaked in distilled H_2O and then 6 × SSC prior to use. The filters were subsequently washed with an additional 5 ml of 6 × SSC. Under these conditions, any closed circular DNA which is nicked assumes a denatured conformation which binds to the membrane filter. The filters were dried and the bound radioactivity was determined.

Exonuclease activity (DNase) determinations were conducted in the standard RNA polymerase reaction mix modified by using [^{3}H]DNA prepared from rat liver as described by Marmur (1961), 0.05 ml of enzyme in TGMED, and excluding the ribonucleoside triphosphates. Incubations were performed for 1 hr at 37° and were stopped by adding 0.1 ml of a solution containing 3.0 M NaCl and 250 mg/ml of calf thymus DNA. Ethanol (0.4 ml) at −20° was then added and the tubes were chilled in ice for at least 10 min. The samples were centrifuged at 2000*g* for 20 min and the resulting supernatant was carefully aspirated and counted directly in a Triton–toluene scintillation fluid.

Assay of Protein Kinase. Protein kinase activity was determined by a modification of the procedure described by Desjardins *et al.* (1972). The reaction mixture contained in 0.060 ml: 0.010 ml of enzyme fraction in TGMED, 0.2 mM EDTA, 0.3 mM EGTA, 5 mg/ml of casein, 0.1 mM [γ-^{32}P]ATP, 2.0 mM magnesium acetate, and 40 mM Tris-HCl (pH 7.0). Incubations were conducted for 30 min at 37° and the incorporation of radioisotope was determined using Cerenkov radiation.

Glycerol Gradient Sedimentation. Analytical zone centrifugation of the enzymes was conducted in 15–30% glycerol gradients containing 0.05 M Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM Na_2EDTA, 0.5 mM DTT, and 0.1 M $(NH_4)_2SO_4$. Fractions from DEAE-Sephadex chromatography were dialyzed against TGMED containing 10% glycerol and 0.1 M $(NH_4)_2SO_4$; 0.4-ml samples were layered on 5.3-ml gradients and centrifuged in a Beckman Spinco SW65 rotor at 64,500 rpm for 4.5 hr; 10-drop fractions (~0.23 ml) were collected and 0.05-ml aliquots were assayed. Sedimentation values were calculated using either bovine catalase (Sigma Chemical Co.) (sedimentation coefficient 11.3 S) or *E. coli* polymerase (14.9 S) as markers.

Isolation and Base Composition of Myeloma DNA. High molecular weight myeloma DNA (single-strand sedimentation coefficient of 33 S) was prepared by a modification of the Marmur procedure (1961). The ethanol precipitated spooled DNA was dissolved in 1 × SSC, treated with boiled RNase (20 μg/ml) and self-digested Pronase (20 μg/ml) for 1 hr each and then deproteinized by using hot phenol (55°). The aqueous phase was reextracted two more times with chloroform–octanol, and the DNA was spooled after the addition of ethanol, dissolved in 0.1 × SSC, and stored over chloroform at 4°.

For the determination of the base composition, myeloma

TABLE I: Enzyme Activity at Different Stages in the Purification through DEAE-Sephadex Chromatography.

Fraction	Protein (mg)	Units[a] (pmol 10^{-1} min at 37°)	Specific Activity[a] (unit mg of protein^{-1})
Nuclei	3600	358,865	99.7
Supernatant (post-sonication)	2518	393,260	156.0
Chromatin pellets	1085	15,960	14.7
Ammonium sulfate pellets	381	313,120	822.0
A 1.5 m pool	153	328,370	2,146.0
DEAE-Sephadex A pool	26	272,982	10,500.0
DEAE-Sephadex B pool	4	47,500	11,900.0

[a] Calculated from average cpm for triplicate assays in the standard polymerase assay (UTP rate limiting) containing native salmon sperm DNA.

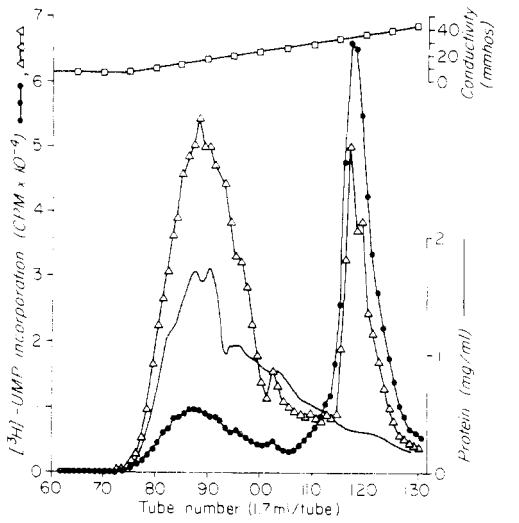

FIGURE 1: DEAE-Sephadex (A-25) column chromatography. Pooled activity from the Bio-Gel A 1.5m chromatography was adsorbed to DEAE-Sephadex at a flow rate of 0.5 ml/min. Following elution with a linear gradient of ammonium sulfate (0.025–0.5 M) in TGMED, duplicate 0.030-ml aliquots were assayed in parallel incubations as described in Methods containing 25 μg of either native (△) or denatured (●) salmon sperm DNA. Other symbols: protein (—); $(NH_4)_2SO_4$ gradient (□).

DNA digested in 70% $HClO_4$ at 100° for 60 min (Marshak and Vogel, 1951); 9 μl of the hydrolysate (containing approximately 10 μg of each base) was spotted on cellulose sheets for thin-layer chromatography and developed in a two-dimensional ascending procedure (Randerath, 1965). The first direction solvent was methanol–hydrochloric acid–water (70:20:10, v/v) and 1-butanol–methanol–water–ammonium hydroxide (60:20:20:1, v/v) was the second direction solvent. The bases were located under uv light, removed from the sheet, and eluted in 5 ml of 0.1 N HCl. The concentration of each base was determined according to Bendich (1957).

Preparation of DNA Templates. Myeloma DNA was prepared from isolated nuclei as previously described. DNA was suspended in either 0.01 M Tris-HCl (pH 8.0) or 0.1 × SSC, and denatured either by heating at 100° for 10 min or by exposure to pH 12.8.

Sucrose Gradient Centrifugation of DNA. The sedimentation values for the DNA templates were determined in 5–20% sucrose gradients under either neutral (0.01 M Tris-HCl (pH 7.4), 1.0 M NaCl–0.001 M Na_2EDTA) or alkaline (0.75 M NaCl, 0.25 M NaOH, 0.001 M Na_2EDTA) conditions. The samples were centrifuged in a Beckman SW40 rotor at 35,000 rpm for 7 hr. Tritiated polyoma DNA forms I and II were used as markers.

Sucrose Gradient Centrifugation of in Vitro Synthesized RNA. Product RNAs were synthesized in 2.0-ml (25 × standard reaction volume) reaction volumes containing 0.1 mM [α-^{32}P]UTP, 0.625 mg/ml of native or denatured myeloma DNA, and 0.625 ml of either polymerase A (following phosphocellulose chromatography) or polymerase B (following DEAE-Sephadex chromatography). After incubation for 60 min at 37°, RNase-free DNase was added to a final concentration of 12.5 μg/ml; the incubation was continued an additional 15 min and stopped by adding EDTA and SDS to 0.01 M and 0.5%, respectively. Unlabeled myeloma RNA was added as carrier (500 μg/ml). The samples were extracted with phenol–SDS at 55° (Scherrer, 1969), precipitated with ethanol, and resuspended in 0.5 ml of 0.01 M Tris-HCl (8.0), 0.05 M NaCl, 0.001 M EDTA, and 5 μg/ml of poly(vinyl sulfate); 0.2-ml samples were layered onto 13.3 ml of 10–40% sucrose gradient containing 0.05 M NaCl, 0.01 M Na_2EDTA (7.0), and 0.2% SDS, and centrifuged in a Beckman SW40 rotor at 35,000 for 15 hr at 25°. The gradients were fractionated into 0.5-ml aliquots; the position of the 18S and 28S myeloma rRNA markers was determined by recording the OD_{254}. High molecular weight yeast RNA was added to each fraction as carrier and the RNA precipitated with an equal volume of 10% Cl_3CCOOH containing 1% sodium pyrophosphate. The samples were filtered through Whatman GF/C filters and washed with 25 ml of 5.0% Cl_3CCOOH plus 0.5% sodium pyrophosphate, dried, and counted in 10 ml of H_2O using Cerenkov radiation.

Chemicals. High molecular weight salmon sperm and calf thymus DNA were obtained from Worthington Chemical Corp., Freehold, N. J. Synthetic polydeoxynucleotides and unlabeled ribonucleoside triphosphates were acquired from P-L Biochemicals. The nonionic detergent Nonidet P-40 was purchased from Shell Oil Co.

Poly([^{3}H]rC), 51.5 μCi/mmol of P, and poly([^{3}H]rA), 50 μCi/mmol of P, were purchased from Miles Laboratories. [^{3}H]UTP (specific activity 1 Ci/mmol) was obtained from Amersham–Searle. [γ-^{32}P]ATP (specific activity 5.6 Ci/mmol) and [α-^{32}P]UTP (specific activity 17.3 Ci/mmol) were purchased from New England Nuclear. Heparin was obtained from Sigma Chemical Co. α-Amanitin was the gift of Professor T. H. Wieland. Rifampicin was the gift of Dr. W. C. Russell, National Institute for Medical Research, Mill Hill, London, England. SV40 and polyoma DNA were kindly provided by Dr. James Champoux, Department of Microbiology, University of Washington.

Results

Isolation Procedures. Enzyme activity at different stages in the purification through DEAE-Sephadex chromatography is illustrated in Table I. Prior to chromatography, RNA polymerase activity is nonlinear with respect to protein concentration making it impossible to give accurate percentage yields: linear dependence on protein concentration is observed only following DEAE-Sephadex chromatography. The elution pattern from

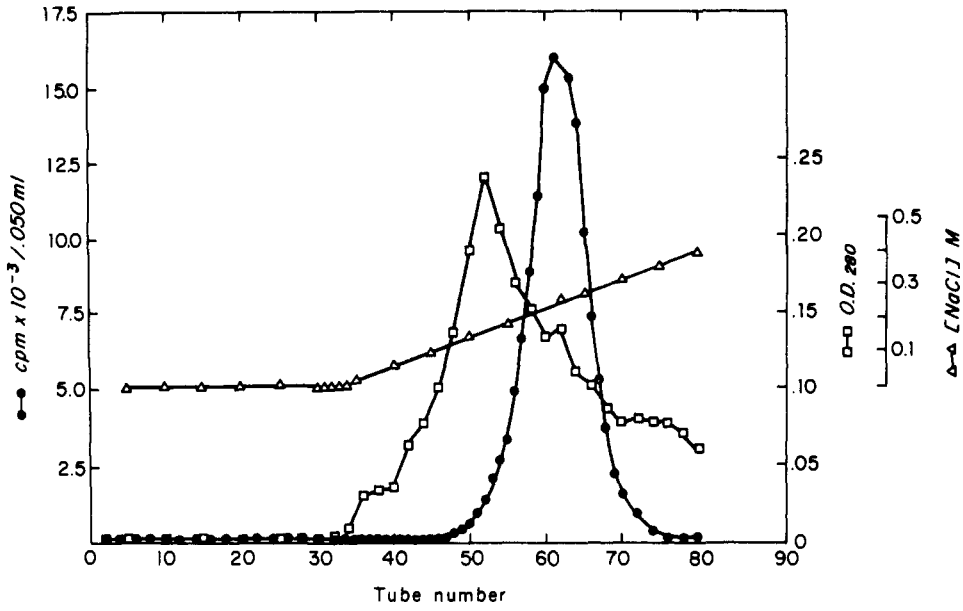

FIGURE 2: Poly(adenylic acid)-Sepharose chromatography. Pooled activity of myeloma polymerase A following DEAE-Sephadex chromatography was desalted on Sephadex G-25 equilibrated in TGED and adsorbed to poly(A)-Sepharose as described in Methods. Fractions containing the flow-through and wash volumes were collected at 5 ml/tube. Fractions of 3.0 ml/tube were collected during the gradient elution and 0.040-ml aliquots were assayed in the standard polymerase assay containing 12.5 μg of native salmon sperm DNA. Symbols: RNA polymerase activity, (●); protein (□); NaCl concentration (△).

DEAE-Sephadex (Figure 1) reveals two major RNA polymerase activities which exhibit marked preferences for native and denatured salmon sperm DNA. The first major species, myeloma RNA polymerase A (MP-A), elutes around 0.12 M $(NH_4)_2SO_4$ and is α-amanitin resistant whereas the second activity, myeloma RNA polymerase B (MP-B), elutes around 0.25 M $(NH_4)_2SO_4$ and is α-amanitin sensitive.

The further purification of MP-A by polyadenylate-Sepharose chromatography is shown in Figure 2. This procedure accomplishes considerable purification with no loss in activity and the adsorbent is resistant to the action of endodeoxyribonuclease activity (in contrast to DNA-cellulose columns) which still contaminates the preparations of MP-A following DEAE-Sephadex chromatography. The bulk of the protein began elution at approximately 0.175 M NaCl while the polymerase A eluted in a sharp peak at about 0.25 M NaCl. Recovery of activity was 100% based on samples of the DEAE-Sephadex pool kept on ice for a comparable period. The specific activity of MP-A following poly(rA)-Sepharose chromatography was 23,300 pmol 10 min^{-1} mg of $protein^{-1}$.

The subsequent purification of MP-A (following poly(A)-Sepharose) and MP-B (DEAE-Sephadex) was accomplished by centrifugation in glycerol-sucrose gradients as described by Sugden and Keller (1973), separating the polymerase activity from the protein kinase. The recovery for MP-B was approximately 100%, and 20–25% for MP-A. The specific activities in the peak tubes for each enzyme were 25,030 and 200,000 pmol 10 min^{-1} mg of $protein^{-1}$ for MPA and −B, respectively.

Enzymatic Properties. Following DEAE-Sephadex chromatography MP-B preparations are free of any detectable DNA or RNA exonuclease activity. Low levels of DNA endonuclease and protein kinase activity (in the presence of added casein) are present, but are removed during sucrose gradient centrifugation. No RNase H was detected in either DEAE-Sephadex or sucrose gradient fractionated MP-B.

Myeloma polymerase A purified through DEAE-Sephadex is free of detectable DNA exonuclease contamination but still contains considerable DNA endonuclease, protein kinase, and a very low level of RNA exonuclease activity. After sucrose gradient centrifugation, MP-A preparations are free of DNA endonuclease and RNase H.

Both MP-A and MP-B following either DEAE-Sephadex or sucrose gradient centrifugation are dependent on added DNA. Both were maximally stimulated in the presence of approximately 0.060 M $(NH_4)_2SO_4$ and neither NaCl nor KCl was effective in substituting for this requirement. Manganese was preferred over magnesium as a divalent cation by both enzymes and produced optimal activity at 3 and 2 mM for MP-A and MP-B, respectively. The manganese effect is dependent on the conformation of the template employed, a broader Mn^{2+} optimum seen with denatured DNA templates, especially with MP-A.

The response of the enzymes to the α-amanitin is comparable to the results obtained with other mammalian polymerases (Kedinger and Chambon, 1972; Lindell *et al.*, 1970). Polymerase A is inhibited only 20–25% at high concentrations (25 μg/ml) whereas MP-B is 95% inhibited. Rifamycin (25 μg/ml) inhibits the activity of both enzymes by approximately 20%.

Velocity sedimentation of the enzymes in glycerol gradients reveals a sedimentation coefficient of 14.5–15.0 S for both MP-A and MP-B using catalase as a standard and 15.5–16.0 S when compared to *E. coli* polymerase. Neither polymerase demonstrated any tendency to aggregate during sedimentation at low ionic strengths (0.025–0.050 M $(NH_4)_2SO_4$) in contrast to published observations with prokaryote polymerase (Berg and Chamberlain, 1970).

Time course experiments conducted under nonlimiting conditions indicated the kinetics of MP-A resembled prokaryote polymerases, but MP-B demonstrated essentially two-stage linear time course kinetics regardless of the DNA template conformation. The reaction with MP-B shows a linear incorporation for 10–12 min, which decreases to a second linear rate of incorporation for the remaining period of incubation. This rate shift is seen with MP-B purified through DEAE-Sephadex and glycerol gradient sedimentation indicating it is a function of the polymerase itself and not the effect of some contaminating

TABLE II: Enzymatic Activity at Polymerases A and B on Defined Polynucleotide Templates.

	Nucleotide Incorporated	Activity Rel to Myeloma DNA[a]	
		Enzyme A	Enzyme B
Poly(dA)	U	0.21	0.05
Poly(dT)	A	0.31	0.05
Poly(dA)·poly(dT)	U	0.16	0.03
	A	0.22	0.05
Poly(dA-T)·poly(dA-T)	U	1.03	3.34
	A	1.11	2.85
Poly(dG)	C	0.08	0.01
Poly(dC)	G	1.61	2.10
Poly(dI)	C	0.07	0.01
Poly(dG)·poly(dC)	G	0.25	0.71
	C	0.04	0.04
Poly(dC)·poly(dI)	G	1.69	13.72[b]
	C	0.08	0.09

[a] Relative activities were calculated as described by Blatti *et al.* (1970). The activities on the polynucleotide templates are expressed relative to the myeloma DNA template and are corrected for the base composition of myeloma DNA. Activities on single-stranded templates are expressed relative to denatured myeloma DNA and the activities on double-stranded templates relative to native myeloma DNA. Base composition of myeloma (MOPC 21) DNA was determined as described in Methods: A, 29.3%; G, 21.2%; C, 21.3%; T, 28.0%. [b] May be nonsaturating for template.

protein. Preincubation of DNA, enzyme, or DNA–enzyme for 20 min at 37° failed to alter this rate shift, thus eliminating the possibility of trivial time-dependent denaturation or inactivation of some component of the reaction.

Template Interactions. Homopolymeric DNA. Myeloma polymerases A and B differ markedly in their ability to transcribe various single- and double-stranded synthetic polydeoxynucleotides (Table II). Polymerase A was relatively inactive on all single-stranded templates except for poly(dC). Although little incorporation was observed with the other single-strand polymers, the level of transcription achieved with the poly(dPyr) strands was uniformly higher than that seen with poly(dPur) templates. Of the double-stranded polymers only poly(dA-T)·poly(dA-T) and poly(dC)·poly(dI) were effective templates producing respectively 1.1 and 1.7 times the rate of incorporation seen with native DNA.

Of the single-stranded polymers used in this study, only poly(dC) showed any appreciable template activity with MP-B. The double-stranded polymer poly(dA-T)·poly(dA-T) was a threefold better template than native DNA while the sequence isomer poly(dA)·poly(dT) was completely inactive. Poly(dC)·poly(dI) was the most permissive template for polymerase B supporting asymmetric transcription into poly(rG) at 13.7 times the rate seen with native DNA.

With the exception of poly(dA)·poly(dT), which was essentially inactive, the remaining poly(dPur)·poly(dPyr) templates were asymmetrically transcribed by both polymerases into poly(rPur). The only poly(dPur-Pyr)·poly(dPur-Pyr) template, poly(dA-T)·poly(dA-T), supported the incorporation of each complementary ribonucleoside monophosphate by both polymerase A and B. Although the stability of several of the hybrid DNA–RNA products is greater than the original DNA–DNA templates, which could effectively inhibit continued transcription (Chamberlin, 1965), their effect, if any, is probably minimal during the 10-min incubation period used in these experiments.

Natural DNAs. The base composition of RNAs synthesized *in vitro* on deproteinized myeloma DNA indicates significant differences (Table III). MP-A transcribed a product RNA containing approximately 58.0% G + C regardless of the conformation of the template. The G + C to A + U ratio (GC/AU) for MP-A product RNA is 1.37:1, differing from myeloma DNA, 42.5% G + C and a GC:AU ratio of 1:1.34. The RNA synthesized by MP-B contains approximately equal amounts of G + C (~48%), regardless of the conformation of the template employed.

In an earlier attempt to satisfy the template preference of both enzymes during chromatography (see Figure 1), assays were conducted with saturating amounts of both n-DNA and d-DNA. The results indicated that both MP-A and -B were preferentially binding to and transcribing only the d-DNA template. Competition experiments were performed using native and denatured salmon sperm DNA, in order to exploit the several-fold preference exhibited by myeloma polymerase A for the native template. When 2 μg of denatured salmon sperm DNA were added to a reaction containing MP-A and a saturating concentration of native DNA ($6 \times K_m$ apparent), the incorporation of [^{3}H]UMP was decreased over 80% (Figure 3a). Further increases in the concentration of d-DNA enhanced the UMP incorporation to a level identical with that seen with saturating amounts of d-DNA alone. The addition of increasing amounts of n-DNA to reactions containing saturating levels of d-DNA had no effect on the rate of incorporation. This result

TABLE III: Base Composition of RNAs Synthesized *in Vitro* on Deproteinized Myeloma DNA.

RNA Polymerase	Template	Base Composition of *in vitro* RNA[b]					
		A (%)	C (%)	G (%)	U (%)	G + C (%)	GC/AU (%)
A	Native myeloma DNA[a]	20	31	26.3	22.5	57.3	1.37
	Denatured myeloma DNA	21.3	28.5	29.6	20.4	58.1	1.39
B	Native myeloma DNA	25	27.4	24.6	22.8	52.0	1.08
	Denatured myeloma DNA	25.5	27.1	23.9	23.3	51.0	1.04

[a] Base composition of myeloma DNA described in Table II. [b] Base compositions of RNA were calculated as follows. Duplicate assays were conducted for each of the four ribonucleoside triphosphates (XTP) which contained one ^{3}H-labeled XTP at 0.5 mM (at identical specific activities) and the other three unlabeled XTPs at 1.0 mM each. The average cpm incorporated for each XTP were summed and the percentage composition for each XTP was then calculated.

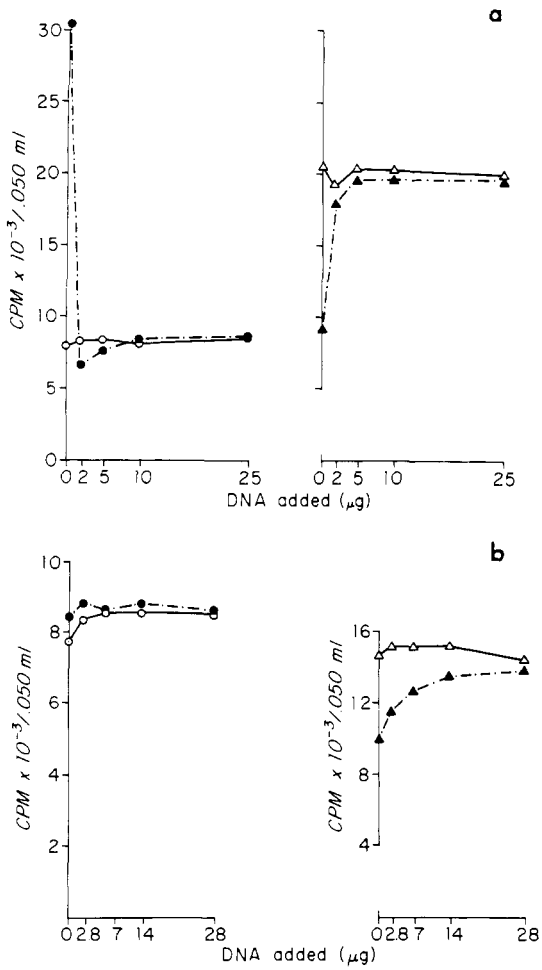

FIGURE 3: (a) Template competition with native and denatured salmon sperm DNA; 20-μl aliquots of DEAE-Sephadex isolated MP-A or -B were used in each standard 0.08-ml reaction. The order of addition was as follows: 25 μg of native (or denatured) DNA was added to the tubes and either 0, 2, 5, 10, or 25 μg of denatured (or native) DNA was then added to the proper tubes; the volumes were adjusted with 0.01 M Tris (pH 7.9); enzyme was added and allowed to stand at room temperature for 5 minutes, the remaining substrates were added, and the incubations were conducted at 37° for 30 min. The $(NH_4)_2SO_4$ concentration was 62.5 mM for both enzymes. Each point represents the average of two separate determinations. Polymerase A (upper left); 25 μg of native plus increasing denatured DNA (● - · - ●), 25 μg of denatured plus increasing native DNA (○ - - - ○). Polymerase B (upper right), 25 μg of native plus increasing denatured DNA (▲ - · - ▲), 25 μg of denatured plus increasing native DNA (△ - - - △). (b) Template competition with native and denatured myeloma DNA; 20-μl aliquots of DEAE-Sephadex isolated MP-A (lower left) or -B (lower right) were assayed as described under Figure 3a modified by using 28 μg of either native or denatured myeloma DNA and increasing concentrations of either denatured or native DNA, respectively. Symbols: saturating native DNA plus increasing denatured DNA (▲ - · - ▲ or ● - · - ●); saturating denatured DNA plus increasing native DNA (○ - - - ○ or △ - - - △).

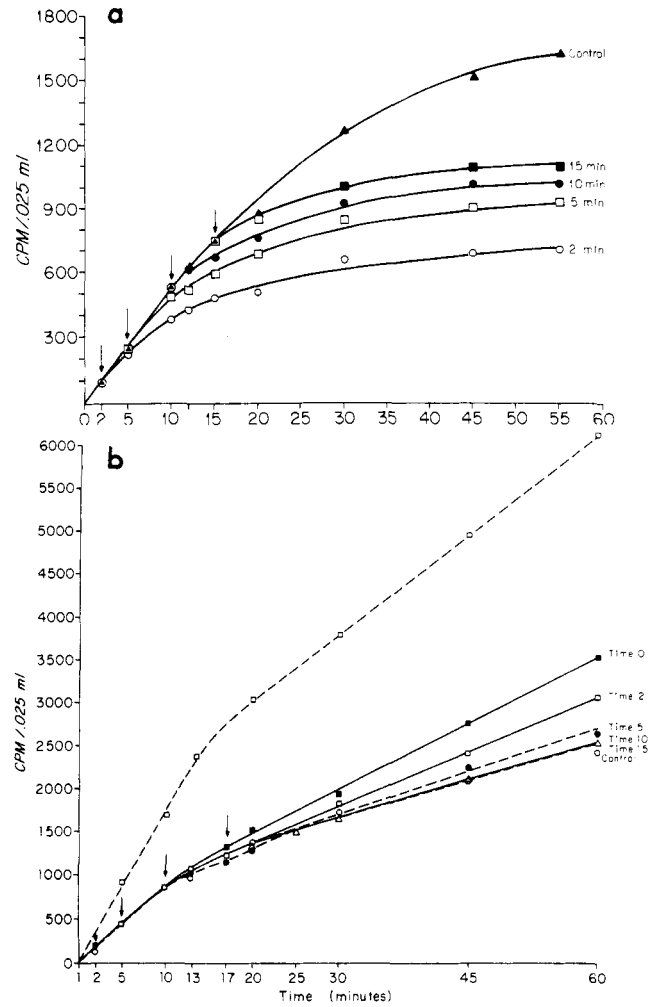

FIGURE 4: (a) Time course DNA competition with polymerase A (upper graph). A 1.6-ml reaction volume (20 × standard assay) contained 0.4 ml of polymerase A, 0.25 mg/ml of native salmon sperm DNA, 0.2 mM [^{3}H]UTP (specific activity 87.5 Ci/mol), and other substrates at standard concentrations. Enzyme and DNA were mixed and allowed to sit at room temperature for 5 min. Then the reaction was started by adding the nucleotide mix to the reaction at 37°, and at 2, 5, 10, and 15 min (arrows) aliquots of appropriate volume were withdrawn and mixed with d-DNA to yield a final d-DNA concentration of 62.5 μg/ml. Duplicate 25-μg/ml aliquots were subsequently assayed at each time point in parallel with the original reaction. $(NH_4)_2SO_4$ concentration was 62.5 mM. Denatured DNA added at 2 min (○ - - - ○), 5 min (□ - - - □), 10 min (● - - - ●), 15 min (■—■); control (▲—▲). (b) Time course competition with polymerase B (lower graph). Reaction conditions were as described for Figure 4a except for use of 0.4 ml of polymerase B. Aliquots were withdrawn and mixed with d-DNA at 0, 2, 5, 10, and 17 min (arrows). The upper curve represents the time course on denatured DNA alone. Denatured DNA added at 0 time (■ - - - ■); 2 min (□ - - - □); 5 min (● - - - ●); 10 and 15 min (△——△); control (○—○); incorporation on denatured DNA (□——□).

demonstrates not only the binding preference of MP-A for denatured DNA but also indicated that the inhibition seen with added d-DNA is not due to some peculiar interaction of template conformations. Assays of MP-B containing n-DNA at saturation showed a 100% increase in incorporation with the addition of 2 μg of d-DNA (Figure 3a). The incorporation continued to increase reaching a maximum equal to that seen with d-DNA alone. Additions of n-DNA to reactions containing saturating d-DNA produced no effect. Although MP-A and -B exhibit optimal incorporation with native and denatured templates, respectively, it appears that when both conformations of template are present in the reaction, both enzymes preferentially bind to and reinitiate RNA chain formation on the denatured template.

To examine the possibility that heat denaturation was altering the primary structure of the DNA (*e.g.*, depurination), a preparation of salmon sperm DNA was divided into two aliquots, one denatured with NaOH and the other by heating. The resulting DNAs appeared equally effective in the inhibition of MP-A.

When myeloma DNA was employed in a similar competition assay the addition of denatured DNA to incubations con-

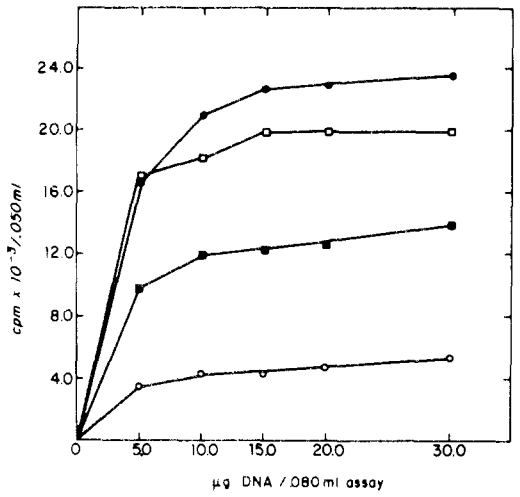

FIGURE 5: Template saturation. Standard polymerase assays were conducted with 0.020 ml of DEAE-Sephadex fractionated enzymes and increasing concentrations of native or heat-denatured salmon sperm DNA. Symbols: MP-A with either native DNA (●), or denatured DNA (○); MP-B with either native DNA (■) or denatured DNA (□).

taining saturating native DNA produced little or no effect on polymerization with MP-A, while the analogous experiments with MP-B yielded identical results with those seen with salmon sperm DNA (Figure 3b).

Time course experiments were designed to determine the degree to which template competition may occur at different stages during active polymerization on native DNA (Figure 4a,b). Samples were withdrawn from a 20-fold reaction volume containing saturating native salmon sperm DNA, and added to tubes containing a predetermined amount of d-DNA to yield a final d-DNA concentration equal to 62.5 μg/ml (5 μg/0.08 ml of reaction volume). Experiments with MP-A indicated that a significant portion of the enzyme was available to bind to the d-DNA as early as 2 min into the incubation, noted by the immediate decrease in the velocity (Figure 4a). The addition of d-DNA to MP-B (Figure 4b) did not demonstrate any increase in the velocity during the first 10–12 min but did show an increase after 15 min, dependent upon the time of addition of the denatured template. These findings suggested that either there was an excess of enzyme *vs.* template in polymerase A incubations (relative to polymerase B reactions) or that polymerase B actually remained bound to the native template in an actively transcribing complex for a longer average period of time. Saturation experiments support the latter contention as both reaction mixes contained at least six times the K_m apparent for native template (Figure 5).

Similar experiments employing heparin as an inhibitor have corroborated this conclusion. Heparin interacts with RNA polymerase inhibiting either free enzyme or DNA bound enzyme not involved in active transcription (Walter *et al.*, 1967; Siefert, 1970b). When added to reactions containing polymerase A, an immediate and relatively constant level of inhibition was observed regardless of the time of addition. Polymerase B reactions were resistant to heparin added early in the incubation, but became increasingly sensitive to its effects at 10–15 min.

Initiation of transcription was examined following the incorporation of [γ-^{32}P]ATP and [^{3}H]UMP plus [^{3}H]CMP. Polymerase A undergoes a rapid rate of initiation (incorporation of γ-^{32}P) in the first 5 min, and then levels off to parallel the rate of polymerization (incorporation of ^{3}H) for the remainder of

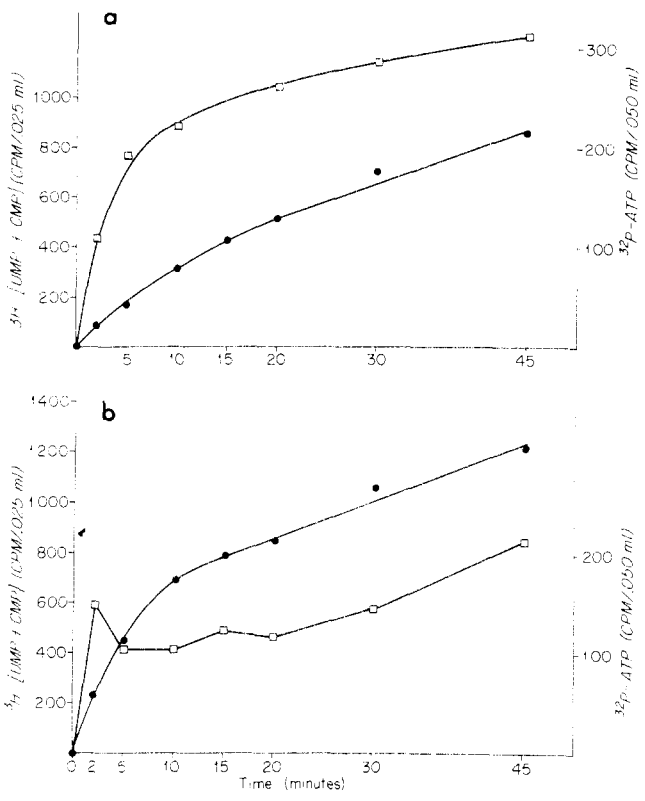

FIGURE 6: (a) Time course of initiation of polymerase A with [γ-^{32}P]ATP (upper graph). The incorporation of [^{3}H]UMP and [^{3}H]CMP was determined by withdrawing 2 × 0.025-ml aliquots at the indicated time points. The reactions contained in 0.4 ml: 0.1 ml of polymerase A, 350 μg/ml of native myeloma DNA, [^{3}H]UTP (specific activity 1 Ci/mmol) and [^{3}H]CTP (specific activity 1 Ci/mmol) at 12.5 μM each, 70 mM $(NH_4)_2SO_4$, and other reagents as indicated in Figure 1. Assays for [γ-^{32}P]ATP incorporation contained in 0.8 ml: 2.25 μM [γ-^{32}P]ATP, UTP, GTP, and CTP at 0.7 mM each, 0.2 ml of either polymerase A or B, 350 μg/ml of native myeloma DNA, 70 mM $(NH_4)_2SO_4$, and other reagents as described in Methods. The incorporation of [γ-^{32}P]ATP was monitored by pipetting 2 × 0.05-ml aliquots at the indicated time points into tubes containing 500 μg of yeast RNA in 0.5 ml of water at 0°. A 0.5-ml aliquot of 0.8 M PCA with 1% PO_4 and 1% PP_i was added and allowed to stand on ice for at least 20 min. The samples were then centrifuged at 1000*g* for 10 min and the pellets washed four times with 0.25 M PCA plus 1.0% sodium phosphate and 1.0% sodium pyrophosphate; 0.5 ml of 0.3 M KOH was added to the pellet and allowed to incubate 8 hr at 37°. The supernatant was collected with 2 × 5 ml washes of H_2O and counted using Cerenkov radiation. Each point is an average of two determinations. Incorporation of [γ-^{32}P]ATP (□); [^{3}H]CMP plus [^{3}H]UMP (●). (b) Time course of initiation by polymerase B with [γ-^{32}P]ATP (lower graph). Conditions were identical with those described in legend to Figure 6a except for the employment of polymerase B. Incorporation of [γ-^{32}]ATP (□); [^{3}H]UMP + CMP (●).

the incubation (Figure 6a). Polymerase B exhibits a rapid burst of initiation in the first 2 min which quickly plateaus, and γ-^{32}P incorporation does not resume until 15 min, when it shows a constant rate parallel with the rate of polymerization (Figure 6b).

The RNAs synthesized on native and denatured homologous myeloma DNA reveal differences in the size of products catalyzed by the two enzymes. MP-A following DEAE-Sephadex was further purified by chromatography on phosphocellulose to eliminate residual endonuclease activity. The bimodal distribution in product size synthesized by polymerase B on native DNA (Figure 7a) is seen to disappear when the template is denatured (Figure 7b) with a loss of the lower molecular weight (7 S) component and an increase in the higher molecular

weight product (10 S). Polymerase A products are small (4–5 S) regardless of the template conformation used (Figure 7a and b).

To examine the possibility that the template size itself limited the product RNA, DNA was subjected to sucrose gradient centrifugation. DNA in alkaline sucrose gradients using [^{3}H]polyoma DNA as marker indicated an average single-stranded sedimentation coefficient of 33 S and 22 S for myeloma and salmon sperm DNA, respectively. It appears that the template size was not a limiting feature.

Discussion

The elution profile from DEAE-Sephadex and the template preferences of DNA dependent RNA polymerases A (I) and B (II) isolated from murine myeloma are analogous to the enzymes prepared from other mammalian sources (Jacob, 1973; Roeder and Rutter, 1970; Kedinger and Chambon, 1972; Sugden and Keller, 1973: Froehner and Bonner, 1973). The specific activity of polymerase B purified through sucrose gradient centrifugation, 199 nmol of [^{3}H]UMP incorporated 10 min^{-1} mg of protein^{-1}, compares very favorably with the specific activity of calf thymus polymerase B similarly purified through glycerol gradient centrifugation (270 nmol units, Kedinger and Chambon, 1972). The significant loss in activity during the sucrose gradient centrifugation of polymerase A even in the presence of protease inhibitor is not presently explicable.

Myeloma polymerases A and B transcribe various polydeoxynucleotide templates (Table II) in a manner similar to *E. coli* RNA polymerase (Wells *et al.*, 1970). The double-stranded polymers of poly(dPyr)·poly(dPur) are transcribed asymmetrically by *E. coli* polymerase and both of the mammalian polymerases into poly(rPur) products. Poly(dC)·poly(dI) promotes the transcription of poly(rG) at a level exceeding that seen with native DNA by 1.7- and 13.7-fold for polymerase A and B, respectively. The importance of the complementary poly(dPur) strand, even though it is not transcribed, is indicated by the six-fold increase in transcription by MP-B over the level seen with the single-stranded poly(dC). Why MP-A fails to demonstrate a comparable increase when the poly(dC) strand is in a double-stranded configuration is unclear, especially in light of the preference of the enzyme for native templates. The dominance of the poly(dPyr) strands, especially poly(dC), is apparent for both mammalian and prokaryotic polymerases.

Our results with myeloma polymerase B are in agreement with a similar polydeoxynucleotide template study conducted with rat liver polymerase B (Blatti *et al.*, 1970). These investigators reported a 13.7-fold incorporation of (rG) with poly(dC)·poly(dI) and a 4.4-fold incorporation with poly(dC) when compared to the incorporation with native rat liver DNA. Incorporation of rG with poly(dC) and poly(dI)·poly(dC) was 4.6 and 13.7, respectively, when both were compared to native DNA. The findings of Lentfer and Lezius (1972) using a preparation of polymerase B isolated from another myeloma cell line conflict with ours in several respects. The reasons for this may be related to the rate-limiting concentrations of ribonucleoside triphosphates employed by these authors, although the basis for the extremely low level of incorporation with poly(dC)·poly(dI) is not known.

It has been proposed (Szybalski *et al.*, 1966) that pyrimidine-rich clusters interspersed in DNA play a significant role in determining the specificity of both the initiation and termination of RNA transcription. This conclusion has been supported by examination of the transcription of synthetic polydeoxynucleotides by *E. coli* RNA polymerase (Morgan, 1970). Our results and similar findings with rat liver polymerases

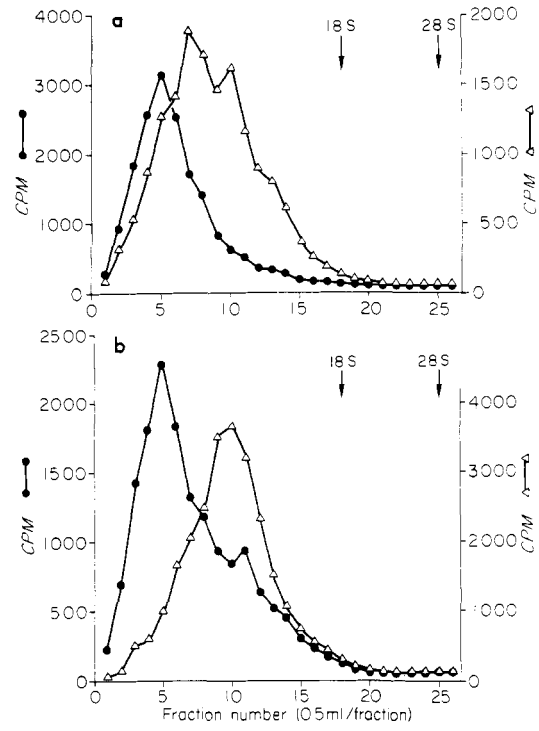

FIGURE 7: (a) Sucrose gradient analysis of *in vitro* synthesized RNA (upper graph). Fractions of 0.2 ml containing 52,800 cpm (polymerase A) or 41,000 cpm (polymerase B) or *in vitro* synthesized ^{32}P-labeled RNA transcribed from native myeloma DNA were layered onto linear 10–40% sucrose gradients and centrifuged as described in Methods. Polymerase A product RNA (●); polymerase B product RNA (△). (b) Sucrose gradient analysis of *in vitro* synthesized RNA (lower graph). Fractions of 0.2 ml containing 46,000 cpm (polymerase A) or 63,000 cpm (polymerase B) of *in vitro* synthesized ^{32}P-labeled RNA transcribed from denatured myeloma DNA were treated as described in Figure 7a. Polymerase A product RNA (●); polymerase B RNA (△).

(Blatti *et al.*, 1970) demonstrate that mammalian RNA polymerases also engage in the asymmetric transcription of the poly(dPyr) strand of poly(dPur)·poly(dPyr) in the same manner as the prokaryote polymerases and exhibit a strong preference for poly(dC) polymers. The dominance of pyrimidine strands in *in vitro* transcription appears applicable to RNA polymerases from widely divergent organisms; however, the actual role these regions play in the transcription of natural DNAs is presently uncertain. Mapping of the poly(rG) sites in bacteriophage λ has confirmed that these sites are located in regions from which transcription occurs, but do not correspond to regions known to function as either promoters or terminators. These sequences may serve instead as "dividers," regulating the production of individual mRNAs from within larger transcriptional units (Champoux and Hogness, 1972).

During transcription of natural DNAs *in vitro*, both myeloma RNA polymerase enzymes undergo release from an actively synthesizing DNA–enzyme–RNA complex and subsequently participate in the reinitiation of new RNA chain formation, but demonstrate significant differences in this context. Polymerase A appears to bind more transiently to native DNA and, following initiation, transcribes only relatively short segments, consequently undergoing multiple reinitiation events throughout the time course of these experiments. This process generates a large and relatively constant proportion of free enzyme which is accessible for binding to either competing d-DNA or heparin. In contrast, neither competing d-DNA nor heparin demonstrably affect the velocity of polymerase B once it has begun the active transcription of native DNA until relatively

late in the time course (10–12 min). This suggests that following the initiation of transcription, the majority of polymerase B molecules remain firmly bound as a synthesizing complex throughout the initial phase of polymerization (*i.e.*, 10–12 min). At this time a major round of reinitiation begins following the release of free enzyme from the DNA–enzyme–RNA complex. The resulting free enzyme then becomes available for binding to a competitor as indicated by the alteration in reaction rates observed after the initial 10-min period. A comparable model has been proposed to explain similar two-stage kinetics observed during the transcription of polydeoxynucleotide templates by *E. coli* polymerase (Morgan, 1970).

Assuming a constant rate of chain elongation for both polymerase enzymes, this suggests that they should synthesize different size product RNAs *in mitro*, and, in fact, this is the case. Zonal centrifugation of the product RNAs of both polymerase A and B synthesized on native homologous myeloma DNA templates (single-stranded 33 S) reveal them to be homogeneous with mean sedimentation values 4–5S for polymerase A products and a bimodal distribution of polymerase B products of approximately 6–7 S and 9–11 S. The absence of significant ribonuclease contamination indicates that formation, and not degradation, is the basis for this difference.[2]

This bimodal distribution of the *in vitro* RNA synthesized by myeloma polymerase B is identical with the pattern reported for RNA synthesized *in vitro* by rat liver polymerase B on large molecular weight DNA (MW $>15 \times 10^6$) containing numerous single-strand "nicks" introduced by limited DNase digestion (Flint *et al.*, 1974). The authors reported that the RNA synthesized on the same size template in the absence of added "nicks" was of much higher molecular weight (800–8000 nucleotides *vs.* 100–800 nucleotides). These results serve to demonstrate the effects that single-strand nicks may produce during *in vitro* transcription. Although the single-stranded molecular weight of the myeloma DNA used in the present study had an average molecular weight of 8.0×10^6 (33 S), there was a considerable range in its size. Consequently, the number of single-strand nicks present in the native DNA may be sufficiently high to generate the relatively diminutive size of the MP-B product RNA, but the different sizes of the product RNAs synthesized by the two polymerases on the same DNA template continue to emphasize the intrinsic differences in their mode of action *in vitro*.

The integrity of the template (nicks, ends, or single-strand regions, etc.) plays a prominent role in determining the different *in vitro* template interactions of the two polymerases. Other investigators have observed that calf thymus AI and B RNA polymerases initiate at different sites on calf thymus DNA but that AI resembles *E. coli* core enzyme in preferentially initiating at . . . "nicks," ends, or denatured DNA regions, whereas the B enzymes initiate on double-stranded sites (Meilhac and Chambon, 1973). More recently, attempts have been made to circumvent the problem of nicks and/or free ends in the template by employing closed circular super-helical DNA from SV40 (Mandel and Chambon, 1974a,b). Although calf thymus enzymes AI and B were both able to transcribe this template the authors reported that their results could be explained by the binding of both polymerases to an unpaired region in the SV40 genome. In fact, they concluded that purified B enzymes from calf thymus and rat liver and calf thymus AI enzyme "are unable to transcribe intact linear double-stranded DNAs of viral or cellular origin" but must rely upon nicks, free ends, or denatured regions in DNA. In this report both of the purified mammalian polymerases seem to resemble *E. coli* core polymerase. Although both myeloma polymerases show a higher affinity for denatured DNA, polymerase A more closely resembles the prokaryote core polymerase by failing to transcribe the denatured DNA as rapidly as the native template, whereas polymerase B prefers the denatured conformation. The apparent stimulation of both polymerases following the introduction of nicks into the template (Flint *et al.*, 1974) also mimics prokaryote core polymerases which can utilize these nicks for new initiation sites, whereas *E. coli* holoenzyme is inhibited by such a template modification (Hinkle *et al.*, 1972).

Whether or not isolated denatured regions may serve as physiological promoter sites *in vitro* is presently unclear. The ability of the isolated RNA polymerases to synthesize RNAs of different size and base composition on identical denatured DNA templates in the absence of any demonstrable RNase contamination serves to indicate the different modes of action exercised by these enzymes *in vitro*. Their selectivity is further evidenced by the distinct preference exhibited by each polymerase for both single- and double-stranded polydeoxynucleotide templates. These data serve to demonstrate the importance of the primary nucleotide sequence as a contributor to the binding process, possibly in the orientation of the polymerase molecule for proper strand selection. Competition experiments between calf thymus AI and B enzymes for binding sites on SV40 DNA "suggest that some of these sites could be different for the two enzymes" (Mandel and Chambon, 1974b). Even though the binding reaction may be shown to occur in the denatured regions of SV40, there would still appear to be some method of site selection specific to each species of polymerase. In light of the present evidence, the regulation of transcription in eukaryotes may be determined by: (1) an inherent template specificity of each species of RNA polymerase and its preference for one of two distinct classes of promoter-like sequences in DNA, and (2) the disposition of specific chromatin proteins which may dictate the accessibility and/or the native–denatured configuration of these promoter regions.

The constraints imposed upon the specificity of *in vitro* transcription by the disposition of the chromatin nucleoproteins have been recently documented using prokaryote polymerase (Astrin, 1973; Axel *et al.*, 1973; Gilmour and Paul, 1973). Other investigators have noted that *E. coli* polymerase seemingly initiates at different sites than do the mammalian enzymes on both DNA (Meilhac and Chambon, 1973) and chromatin (Keshgegian *et al.*, 1973). The mechanism whereby *E. coli* polymerase manages to maintain transcriptional fidelity while apparently utilizing different sites for chain initiation presents a provocative question. The demonstration of specific messenger sequences in RNA transcribed from chromatin by prokaryote polymerase could be generated by a process of random initiation with subsequent transcription through specific gene loci. This process would obviate the necessity for specific initiation by the prokaryote enzyme while accomplishing the transcription of the specific gene products which have to date been used as molecular probes. This model receives direct confirmation from the work of Cedar and Felsenfeld (1973) which demonstrates that each prokaryote polymerase molecule that binds to eukaryote chromatin subsequently undergoes initiation and chain elongation. An obvious inference is that while both eukaryotic and prokaryotic RNA polymerases may exercise similar modes of strand selection, they do not respond to similar control mechanisms. Thus it seems that any study of the regulation of transcription in eukaryotes will necessitate the

[2] The authors recognize a critical analysis of potential endonucleolytic RNA alterations can be carried out under denaturing conditions (Me_2SO or formamide gradients), and such studies are under way.

use of well-characterized homologous RNA polymerase enzymes and templates (DNA and chromatin) before meaningful conclusions can be drawn.

Acknowledgments

The authors are indebted to Dr. James Champoux for the donation of tritiated polyoma DNA and for his suggestions and help with the DNA endonuclease assay. We thank Drs. Robert Krueger and Ursula Storb for providing us with the seed cultures of myeloma used in this study.

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