DNA-Dependent RNA Polymerases I and II from Kidney. Effect of Polyamines on the in Vitro Transcription of DNA and Chromatin†

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ABSTRACT: DNA-dependent RNA polymerases I and II were purified from pig kidney nuclei by chromatography on DEAE-Sephadex and phosphocellulose. When nonlimiting amounts of double-stranded DNA were used as the template, the in vitro transcription was markedly stimulated by spermidine and spermine. Maximal stimulation of RNA polymerase I occurred at 2–5 m*M* spermidine and 0.5–2 m*M* spermine, whereas optimal polyamine concentrations for RNA polymerase II were 5–10 and 1–5 m*M* for spermidine and spermine, respectively. DNA transcription by polymerase II was stimulated to a greater extent than that of polymerase I. Higher spermine (5–10 m*M*) concentrations were strong inhibitors of both polymerases under these conditions. The apparent K_m of RNA polymerases I and II for UTP was unchanged at optimal polyamine concentration; under the same conditions the maximal reaction velocity was increased two- to three-fold and was essentially due to an increase in the rate of chain elongation. Thus, in a typical experiment the average chain length as determined by the UMP/uridine ratio increased from 570 to 1330 and the chain elongation rate increased from 0.64 to 1.44 nucleotides × sec^{-1} in the presence of spermine. When limiting quantities of native DNA were employed as the template, both RNA polymerases I and II were inhibited by 1–2 m*M* spermine. Kidney chromatin could be transcribed by homologous RNA polymerases with an efficiency ranging from 2 to 10% of that with native DNA. When chromatin was used in nonlimiting amounts instead of DNA, RNA polymerase II activity was again stimulated about two-fold at 2 m*M* spermine. Under these conditions, RNA polymerase I activity was inhibited by spermine. The inhibition of RNA synthesis in vitro at limiting quantities of templates (DNA or chromatin) could be overcome by preincubation of the enzyme with templates before polyamines were added. This inhibition thus appears to be due to a block in the initiation of RNA chains. Similar inhibition of transcription by RNA polymerase II was also observed with limiting quantities of chromatin as the template.

The existence of multiple forms of DNA-dependent RNA polymerases[1] is well established (for recent reviews, see Jacob, 1973; Chambon et al., 1974). Factors which stimulate RNA polymerase II have been reported in calf thymus (Stein and Hausen, 1970), rat liver (Seifart, 1970; Seifart et al., 1973), mouse myeloma (Lentfer and Lezius, 1972), HeLa and KB cells (Sugden and Keller, 1973), and ascites cells (Lee and Dahmus, 1973). Similar factors, also protein in nature, have been reported for RNA polymerase I from rat liver (Higashinakagawa et al., 1972) and ascites tumor (Froehner and Bonner, 1973). It is also well known that both prokaryotic and eukaryotic RNA polymerase activities may be regulated by smaller nonprotein molecules (see Burgess, 1971; Jacob, 1973; Chambon et al., 1974). Of these compounds, two naturally occurring polyamines, spermidine and spermine, have attracted a great deal of attention over the past few years, especially following reports that they are possibly involved in the regulation of tumor growth (see Russell, 1973) and in the action of several hormones, including steroids (Caldarera et al., 1968; Raina and Jänne, 1970; Williams-Ashman and Lockwood, 1970; Williams-Ashman and Reddi, 1971; Oka, 1974; Oka and Perry, 1974). Moreover, polyamine levels in hormone responsive tissues have been shown to fluctuate according to the hormonal status of the cell, i.e. increase with a hormonal stimulus and decline following the withdrawal of the hormone (Moulton and Leonard, 1969; Pegg et al., 1970; Cohen et al., 1970; Russell and Taylor, 1971). Polyamines have been reported to stimulate RNA synthesis in isolated nucleoli (Caldarera and Moruzzi, 1970; Raina and Jänne, 1970; Gfeller et al., 1973), the activity of crude or partially purified mammalian RNA polymerases from rat liver (Stirpe and Novello, 1970) and beef brain (Singh and Sung, 1972), and the activity of RNA polymerases I and II from calf thymus (Mandel and Chambon, 1974). In none of these studies, however, has the actual mechanism by which polyamines modify the transcriptional process been established.

Over the past few years we have been interested in elucidating the molecular mechanism of the action of steroid hormones on transcriptional events (Jacob et al., 1969, 1975a,b; Sajdel and Jacob, 1971). The present series of investigations is a continuation of these studies and was delineated to clarify mechanisms involved in hormonal regulation of renal RNA polymerases. Since the levels of polyamines appear to be regulated by hormonal variations and may thus participate in mediation of hormone action on gene transcription, we thought it pertinent to investigate the direct effects of polyamines on the in vitro reaction catalyzed by renal RNA polymerases I and II. In the present study we describe the actions of putrescine, spermidine, and spermine on the transcription of native DNA and kidney chromatin by homologous RNA polymerases I and II.

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[1] Abbreviations used are: DNA-dependent RNA polymerase, deoxyribonucleic acid dependent ribonucleic acid polymerase, nucleosidetriphosphate:RNA nucleotidyltransferase (EC 2.7.7.6); Cl_3CCOOH, trichloroacetic acid.

Materials and Methods

Buffers. TGMED buffer contained 50 m*M* Tris-HCl (pH 7.9 at 20°), 25% glycerol (v/v), 5 m*M* $MgCl_2$, 0.1 m*M* Na_2EDTA, and 0.5 m*M* dithiothreitol. TED buffer contained 50 m*M* Tris-HCl (pH 7.9 at 20°), 0.1 m*M* Na_2EDTA, and 2 m*M* dithiothreitol. TGED-30 and TGED-50 buffers contained in addition to TED, 30 and 50% glycerol (v/v), respectively. Dithiothreitol was added to the buffers just prior to use.

Isolation of Nuclei. Fresh pig kidneys were obtained from a local slaughterhouse and transported in crushed ice to the laboratory. The processing of the tissue began within 60 min after the sacrifice of the animals. Nuclei were isolated essentially as described by Busch et al. (1967), using 2.0 *M* sucrose containing 10 m*M* $MgCl_2$ and no spermine for the initial homogenization of the tissue. The homogenate was centrifuged at 50,000*g* for 60 min and the resulting nuclear pellets suspended in TGED-30 buffer. Nuclei isolated by this procedure have been successfully used for the extraction of mammalian RNA polymerases (Jacob et al., 1970; Roeder and Rutter, 1970).

Solubilization of Nuclear RNA Polymerases. Kidney nuclear RNA polymerases were solubilized and purified by successive precipitations with ammonium sulfate and protamine sulfate using a modification of the method described by Kedinger et al. (1972) for calf-thymus enzymes. Usually, four batches of nuclei (each from 300 g of pig kidney tissue) were suspended separately in 0.5 vol (milliliters per gram of original tissue wet weight) of TGED-30 buffer. The homogenates were then made 0.7 *M* with respect to ammonium sulfate by adding a saturated solution (4 *M*, pH 7.9) under continuous stirring with a glass rod. The viscous solution was sonicated (Branson Model W 140 sonifier at full output) 6–8 times for 15 sec followed by 15-sec cooling periods in between until a great reduction of the viscosity occurred. After the sonication, the samples were pooled and freed of insoluble nuclear residue by centrifugation at 50,000*g* for 30 min. The supernatant was brought to 60% fractional saturation with solid ammonium sulfate (enzyme grade, Schwarz/Mann), which was added over a period of 30 min at 0° under continuous stirring. Following an additional stirring for 45 min, the precipitate was removed by centrifugation at 100,000*g* for 120 min. The pellets were taken up in 0.2 vol of TGED-50 and stored at −90° until further processing.

The enzyme preparation in TGED-50 was diluted with TGED-30 until the ammonium sulfate concentration was less than 0.1 *M*. Following dilution, nucleic acids and nucleoproteins were removed by protamine sulfate precipitation. Protamine sulfate (1% aqueous solution, pH 5.5) was added dropwise to the enzyme solution at 0° under continuous stirring to bring the final protamine sulfate concentration to 0.05%. The mixture was allowed to stir for an additional 45 min; the precipitate was then spun down at 100,000*g* for 120 min and discarded. The supernatant containing RNA polymerase activities was concentrated by repeating the ammonium sulfate precipitation step described above. After centrifugation, the pellets were dissolved in 0.1 vol of TGED-50 and the enzyme solution stored at −90°, if not processed immediately.

Chromatography on DEAE-Sephadex (A-25). The enzyme solution was first diluted with TGMED buffer until the ammonium sulfate was 0.03 *M* and then stirred for 60 min with 60 g of DEAE-Sephadex (dry weight) previously equilibrated with TGMED containing 0.03 *M* $(NH_4)_2SO_4$. The slurry was poured into a column (i.d. 5 cm) containing a 5-cm layer of fresh DEAE-Sephadex in TGMED–0.03 *M* $(NH_4)_2SO_4$ buffer. After settling, the column (20 × 5 cm) was washed with 1.5 bed volumes of TGMED–0.03 *M* $(NH_4)_2SO_4$ buffer and the RNA polymerases were eluted using a stepwise gradient comprising one bed volume of each of 0.14 *M* $(NH_4)_2SO_4$ and 0.4 *M* $(NH_4)_2SO_4$ in TGMED buffer. Five-milliliter fractions were collected in tubes containing 0.5 ml of glycerol. From every alternate fraction 0.1 ml was assayed for RNA polymerase activity as described below. Fractions containing RNA polymerase I and II activities were pooled and dialyzed separately overnight at 0° against 20 vol of TGED-30 buffer containing 0.03 *M* $(NH_4)_2SO_4$.

Chromatography on Phosphocellulose. The dialyzed DEAE-Sephadex enzyme preparations were loaded onto phosphocellulose columns (dimensions: 2.5 × 11 cm and 2.5 × 12.5 cm for RNA polymerases I and II, respectively) equilibrated with TGED-30 buffer containing 0.03 *M* $(NH_4)_2SO_4$. Each column was washed with one bed volume of TGED-30–0.03 *M* $(NH_4)_2SO_4$ buffer and the enzymes were then eluted with a linear 0.03–0.6 *M* ammonium sulfate gradient in TGED-30 buffer (total gradient volume: 3 column bed volumes). Three-milliliter fractions were collected in tubes containing 0.3 ml of glycerol, and 0.1 ml from each fraction was taken for enzyme assay. The most active fractions were pooled and glycerol and bovine serum albumin were added to bring the final concentrations to 50% and 1 mg/ml, respectively. The enzyme preparations were stored at −90° until used.

Assay of DNA-Dependent RNA Polymerase. The standard RNA polymerase assay mixture contained in a total volume of 0.4 ml: 100 m*M* Tris-HCl (pH 8.0 at 20°), 2 m*M* $MnCl_2$, 1.3 m*M* NaF, 0.5 m*M* each of ATP, CTP, and GTP, 0.035 m*M* [5,6-^{3}H]UTP (10–20 × 10^3 counts × min^{-1} × $nmol^{-1}$), 10–25 μg of native double-stranded calf-thymus DNA, and 0.05–0.1 ml of enzyme preparation. The final ammonium sulfate concentrations were 30 and 80 m*M* in the assays of RNA polymerases I and II, respectively. When chromatin was used as template, assay conditions were the same except that [^{3}H]UTP was replaced by 0.015 m*M* [^{14}C]UTP (10–20 × 10^3 cpm $nmol^{-1}$). The amount of chromatin used corresponded to 20–80 μg of DNA/tube. Samples were incubated at 37° for 15 min, the reactions terminated, and the amount of UMP incorporated into RNA measured as previously described (Sajdel and Jacob, 1971). One unit of activity was defined as 1 nmol of UMP incorporated into RNA under these conditions.

Initiation of transcription was examined with an assay mixture containing in a total volume of 0.13 ml: 100 m*M* Tris-HCl (pH 8.0 at 20°), 2 m*M* $MnCl_2$, 1.3 m*M* NaF, 0.5 m*M* each of UTP, CTP, and GTP, 0.035 m*M* [γ-^{32}P]ATP (about 10^3 cpm $pmol^{-1}$), 7–10 μg of native calf-thymus DNA, and 0.01–0.02 ml of enzyme preparation. Simultaneous measurement of RNA chain elongation was carried out with identical samples where [2,8-^{3}H]ATP (0.035 m*M*, 10–20 × 10^3 cpm $nmol^{-1}$) replaced [γ-^{32}P]ATP. After incubation for various times at 37°, the reaction was terminated by addition of cold ATP (10 μg/tube) and rapid cooling in ice. The sample (0.1 ml) was then pipetted onto DEAE-cellulose disks (Whatman DE 81) which were washed and counted for radioactivity essentially as described by Blatti et al. (1970). Alternatively, and to ensure that the counts recovered with [γ-^{32}P]ATP represented incorporation into RNA and not into proteins, the reaction

tubes were heated following incubation for 2 min at 100° to destroy RNA polymerase activity and then cooled in ice. Thereafter, α-chymotrypsin (Sigma, 200 μg) or proteinase K (Merck, 100 μg) was added into each tube, which were incubated for an additional 20 min at 37°. The reaction was stopped by pipetting the samples onto DEAE-cellulose disks, as described above.

Measurement of the Average RNA Chain Length. Incubations were carried out using a standard assay mixture with the exception that [^{3}H]UTP was replaced by the same amount of [^{14}C]UTP. Enough reaction tubes were run simultaneously to obtain 10–20 × 10^4 cpm of [^{14}C]UMP incorporated in the pooled product. The labeled RNA was precipitated with ice-cold 10% Cl_3CCOOH containing 0.04 *M* sodium pyrophosphate and pelleted by centrifugation at 30,000*g* for 15 min. Following washes of the pellet with 5% and 2.5% Cl_3CCOOH–pyrophosphate solutions, the pellet was taken up in 0.3 *N* KOH and hydrolyzed at 37° for 18 hr. The sample was neutralized with 70% perchloric acid, insoluble potassium perchlorate spun down, and clear supernatant lyophilized. The dry residue was taken up in a minimal volume of distilled water and applied onto a poly(ethylenimine)-cellulose thin-layer plate. The separation of uridine 3′-phosphate and uridine was performed essentially as described by Randerath and Randerath (1967), using 1 *M* LiCl as the solvent. Uridine and uridine 3′-phosphate were localized on the thin-layer plate with markers run simultaneously with the samples and scraped off and the radioactivity measured. The ratio of ^{14}C radioactivity in the internal uridine 3′-phosphate to terminal uridine gave the average chain length of the RNA formed.

Estimation of the Size of the RNA Synthesized in Vitro. The reactions were performed with the standard assay mixture and stopped by addition of 1 ml of 0.05 *M* sodium acetate buffer (pH 5.1) containing 0.3% sodium dodecyl sulfate and 0.14 *M* NaCl. ^{3}H-Labeled RNA was extracted with water-saturated phenol and precipitated with 2.5 vol of cold ethanol essentially as described by Jacob et al. (1967). The precipitate was collected by centrifugation at 40,000*g* for 15 min and dissolved in a small volume of a buffer composed of 1 m*M* Tris-HCl (pH 7.3), 0.03 *M* LiCl, 0.5 m*M* EDTA, and 0.03% sodium dodecyl sulfate and heated for 2 min at 80°. Samples (0.2 ml) were then layered onto 10–30% sucrose density gradients and centrifuged at 20° for 3.5 hr at 58,000 rpm in a Spinco SW 60 rotor (Sugden and Keller, 1973). Tritium-labeled ribosomal RNA from rat liver and *Escherichia coli* transfer RNA (Schwarz/Mann) were run as markers in parallel gradients. Fractions (0.15 ml) were collected from the bottom of the tube and 0.1 ml from each fraction was applied onto DEAE-cellulose filter disks which were washed and counted for radioactivity as described by Blatti et al. (1970).

Preparation of Templates. Highly polymerized calf-thymus DNA (Sigma, St. Louis, Mo.) was dissolved by gentle shaking in 3 m*M* NaCl (1 mg of DNA/ml). DNA was denatured by heating at 100° for 2 min followed by rapid cooling in ice-water.

Kidney chromatin was prepared from nuclei isolated by the heavy sucrose method (see above). Nuclei were washed once with 2 vol of 0.32 *M* sucrose containing 2 m*M* $MgCl_2$ and 0.2% Triton X-100 and twice with 3 vol of 75 m*M* NaCl–24 m*M* EDTA (pH 8.0) using a loosely fitting Dounce homogenizer. Between each wash the chromatin was spun down at 15,000*g* for 15 min. The chromatin was suspended in 3 vol of 10 m*M* Tris-HCl (pH 8.0), homogenized with a tightly fitting Dounce homogenizer, and stirred gently at +4° for 60 min. The swollen chromatin was pelleted at 15,000*g* for 15 min and homogenized in 1.7 *M* sucrose–10 m*M* Tris-HCl (pH 8.0). This homogenate was layered over the same 1.7 *M* sucrose solution and centrifuged in a Beckman SW 27 rotor at 25,000 rpm for 2 hr. The gelatinous chromatin pellets were taken up in 1 vol of 0.4 m*M* Tris-HCl (pH 8.0), homogenized gently, and stored overnight at 4°. Chromatin was diluted the next day to give a concentration of 0.5–1.0 mg of DNA/ml and used within the following 24 hr. Chromatin prepared by this procedure has in our hands produced consistently reproducible results when transcribed with homologous RNA polymerases. The protein:DNA ratio of the chromatin preparation obtained was 1.5–2.0:1 in various preparations.

Chemical Determinations. DNA was determined according to Burton (1956). Protein measurements were usually carried out by the method of Lowry et al. (1951). For estimation of smaller quantities of protein present in purified enzyme preparations, the method of Schaffner and Weissman (1973) was used.

Chemicals. Putrescine, spermidine, and spermine, all as chlorides, were purchased from Sigma, St. Louis, Mo. Polyamine solutions were neutralized prior to use with 0.1 *N* NaOH. α-Amanitin and nonlabeled ribonucleoside triphosphates were purchased from Calbiochem, San Diego, Calif. [2,8-^{3}H]ATP, [γ-^{32}P]ATP, [^{14}C]UTP, and [5,6-^{3}H]UTP were obtained from New England Nuclear, Boston, Mass.

Results

Purification and Properties of Pig Kidney RNA Polymerases I and II. The solubilization and purification procedure for RNA polymerases I and II adopted in this study was intended for large scale preparation of the enzymes from kidney. No attempt was made to isolate and purify the other forms of RNA polymerases (IV or IA and III) known to be present in other mammalian tissues (Jacob, 1973; Chambon et al., 1974; Weinmann and Roeder, 1974).

Table I summarizes the various steps in purification of RNA polymerases from pig kidney nuclei isolated from 1000 g of tissue. Typical elution profiles of RNA polymerase I and II activities from DEAE-Sephadex and phosphocellulose columns are shown in Figures 1 and 2. These fractionations resulted in a 250- to 400-fold increase in specific activity of both RNA polymerases over that obtained following nuclear sonication. The relatively high yield (25–30%) of enzyme activities was attributed to the shorter period of time needed for batchwise adsorption along with a stepwise elution of the enzymes in the DEAE-Sephadex step. Standard column loading technique and/or gradient salt elution resulted in markedly reduced yield of both enzyme activities, possibly owing to greater dilution of the enzymes and longer time needed for their loading onto and elution from the column. The specific activities of kidney RNA polymerases I and II after phosphocellulose chromatography were of the same order of magnitude as those of enzymes purified from other sources by similar procedures (Weaver et al., 1971; Gissinger and Chambon, 1972; Kedinger and Chambon, 1972; Houghton and Cox, 1974; Hall and Smuckler, 1974). More comprehensive purification and characterization of kidney RNA polymerases are currently in progress and will be the subject of a later paper.

The column elution properties of the two renal RNA polymerase activities resolved by this purification technique

Table I: Summary of the Purification of RNA Polymerases I and II from Pig Kidney Nuclei.[a]

Fraction	Protein (mg)		Total Units		Sp Act. (Units/mg of Protein)	
	I[b]	II	I	II	I	II
Post-sonication supernatant	2772		166.3[c]	158.6[c]	0.06	0.08
Ammonium sulfate–protamine sulfate	294		126.4[c]	177.0[c]	0.44	0.60
DEAE-Sephadex	30	15.8	120.8	336.8	4.0	21.3
Phosphocellulose	3.75	3.0	52.0	102.0	13.9	34.0

[a] Enzyme activity was measured in triplicate as described in the text. The activities of RNA polymerases I and II present in post-sonication supernatant and ammonium sulfate–protamine sulfate fractions were determined by their sensitivity to low concentrations (1.25 μg/ml) of α-amanitin (see text). One unit of activity was defined as 1 nmol of UMP incorporated at 37° in 15 min under the standard assay conditions (see Materials and Methods). [b] I = RNA polymerase I; II = RNA polymerase II. [c] These values have been probably underestimated owing to the presence of inhibitory contaminants and product degrading enzymes. Moreover, protamine sulfate inhibited to some extent both RNA polymerases.

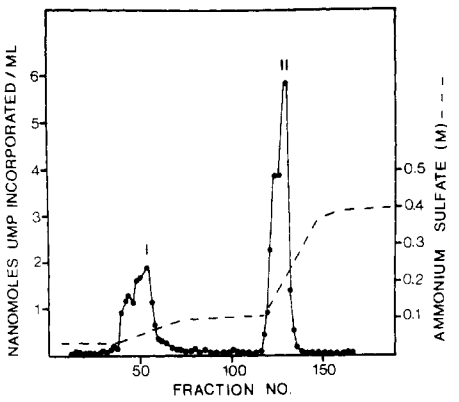

FIGURE 1: Chromatography of pig kidney nuclear DNA-dependent RNA polymerases on DEAE-Sephadex (A-25). The enzyme preparation precipitated with ammonium sulfate and protamine sulfate was diluted with TGMED buffer (total volume 600 ml) and stirred with the resin as described in the text. The sample was allowed to run through the column, whereafter the column was washed with 600 ml of TGMED–0.03 *M* $(NH_4)_2SO_4$ at a flow rate of 5 ml/min. RNA polymerases were eluted at the same flow rate with 400 ml of 0.14 *M* $(NH_4)_2SO_4$ followed by 400 ml of 0.4 *M* $(NH_4)_2SO_4$ in TGMED buffer. Five-milliliter fractions were collected and 0.1-ml aliquots assayed for RNA polymerase activity as described in the text (flow-through and wash fractions are not shown in the figure).

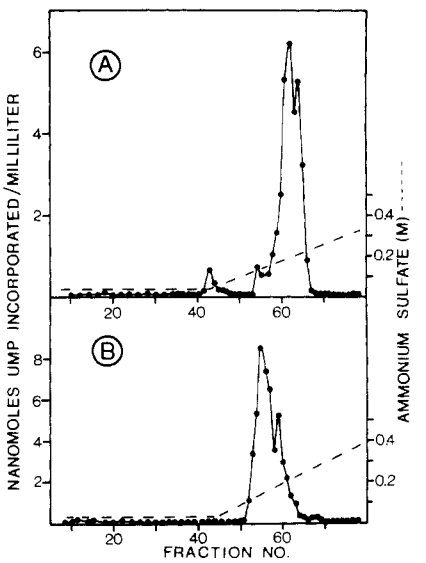

FIGURE 2: Phosphocellulose chromatography. Pooled activities of kidney RNA polymerase I (A, upper graph) and polymerase II (B, lower graph) from DEAE-Sephadex chromatography were dialyzed overnight against TGED-30 buffer containing 0.03 *M* $(NH_4)_2SO_4$ and adsorbed to phosphocellulose columns. Each column was washed with 60 ml of TGED–0.03 *M* $(NH_4)_2SO_4$ and then eluted with a linear 0.03–0.6 *M* $(NH_4)_2SO_4$ gradient in TGED-30 buffer at a flow rate of 2 ml/min. Aliquots (0.1 ml) from the fractions (3 ml each) were assayed for enzyme activity as described in the text.

are consistent with the idea that they represent RNA polymerases I and II. This was further evidenced by their enzymological properties (template preferences, salt optima, optimal divalent cation concentrations) which were characteristic of those reported for other mammalian RNA polymerases I and II (see Jacob, 1973). Moreover, kidney RNA polymerase I was insensitive to α-amanitin (1.25 μg/ml), whereas polymerase II was almost completely (95%) inhibited with this concentration of the toxin, which should not, however, inhibit RNA polymerase III if present in the preparation (Weinmann and Roeder, 1974). Neither RNA polymerase I nor II contained any measurable ribonuclease activity after the purification on phosphocellulose.

Effect of Polyamines on the Transcription of DNA. Both naturally occurring polyamines spermidine and spermine markedly stimulated the formation of labeled RNA when nonlimiting quantities of native DNA were transcribed by kidney RNA polymerase I or II. Putrescine also stimulated RNA synthesis but to a much smaller extent. The results of one typical experiment are shown in Figure 3. In all, four different DNA templates and six different enzyme preparations over a concentration range of 0.01–0.4 unit/tube were used in these studies. No significant changes were found in the optimal stimulatory polyamine concentrations from one experiment to another, when nonlimiting quantities of DNA template were used.

For the maximal stimulation of DNA transcription to occur, the following polyamine concentrations were needed: 2–5 m*M* spermidine and 0.5–2 m*M* spermine for polymerase I and 5–10 m*M* spermidine and 1–5 m*M* spermine for polymerase II. In addition to different optimal polyamine concentrations, the relative stimulation of transcription caused by polyamines seemed to be different for the two renal polymerases. Thus, polymerase II was always more markedly activated by polyamines (Figure 3). At higher polyamine (spermine) concentrations there was a definitive decrease in the degree of stimulation of transcription; in fact, 5–10 m*M* spermine inhibited almost completely polymerase I activity. Kidney RNA polymerases I and II

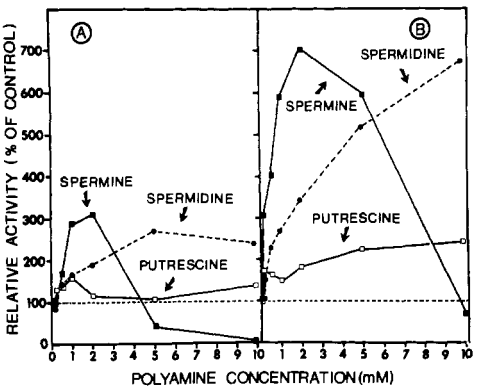

FIGURE 3: Effect of various putrescine, spermidine, and spermine concentrations on the DNA transcription by kidney RNA polymerase I (A, left graph) and polymerase II (B, right graph) activities. Enzyme assays were conducted in triplicate under standard incubation conditions (Materials and Methods) using 25 μg of calf-thymus DNA and 0.12 and 0.20 unit of kidney RNA polymerases I and II, respectively. The values are expressed as relative to controls (= 100, no polyamine).

seemed, therefore, to differ also in their sensitivity toward high polyamine concentration. At optimal spermine concentration, other polyamines (spermidine and putrescine) did not potentiate its action when added over a concentration range of 0.5–5 mM (results not shown), suggesting that polyamines act by the same mechanism. For this reason, the most potent polyamine spermine was chosen as the model compound and was used in most of the subsequent studies.

In the case of kidney polymerase II it was of interest to observe that spermine retained at least part of its stimulatory action when native double-stranded DNA template was replaced by denatured DNA, although the extent of stimulation was considerably reduced from that obtained with native DNA (Table II). The optimal spermine concentration in the presence of denatured DNA was similar to that found for the transcription of native DNA. In this respect, mammalian polymerase II seems to differ from prokaryotic RNA polymerase, which was not stimulated by polyamines with denatured DNA as the template (Abraham, 1968).

Enzyme Kinetic Parameters. In order to determine the mechanism by which polyamines stimulated the reaction catalyzed by renal RNA polymerases, we first attempted to reveal the component of the reaction with which polyamines interacted. To this end, we determined some enzyme kinetic properties of the kidney polymerases I and II in the presence and absence of spermine and spermidine. These studies are summarized in Figure 4, which shows that polyamines do not change significantly the apparent K_m of the enzymes for their ribonucleoside triphosphate substrates. Thus, the K_m of the polymerase I for UTP was 23 μM in the absence of spermine and 20 μM in 1 mM spermine concentration. Similarly, the K_m of polymerase II for UTP was 29 μM in the absence of polyamine, and 29 and 26 μM in 2 mM spermine and 10 mM spermidine concentrations, respectively. Polyamines markedly increased the maximal velocity of the reaction; for example, the increases in this particular experiment were as follows: polymerase I activity increased from 0.71 to 1.25 nmol of UMP incorporated (1 mM spermine) and polymerase II from 0.24 to 0.8 (2 mM spermine) or to 0.67 nmol of UMP incorporated (10 mM spermidine). These results suggest that polyamines do not interact with the enzyme–substrate complex or change the affinity of the enzyme for ribonucleoside triphosphates, but probably exert their action via interference with the template.

Table II: Effect of Spermine on the in Vitro Transcription of Denatured Calf-Thymus DNA by RNA Polymerase II.[a]

[Spermine] (mM)	[^{3}H]UMP Incorpd (nmol)	% of Control
0	0.36	100
0.1	0.38	106
0.25	0.37	103
0.5	0.51	142
1.0	0.52	144
2.0	0.59	164
5.0	0.46	128
10.0	0.21	58

[a] Enzyme assay was run under standard assay conditions using 25 μg of DNA denatured as described in the text. One-tenth unit of pig kidney RNA polymerase II was used in each tube. Values are means of triplicate assays.

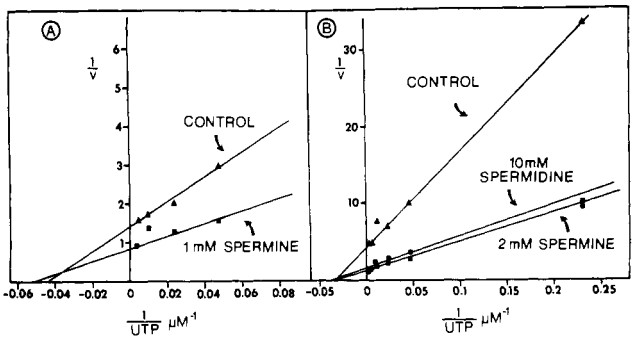

FIGURE 4: Kinetics of RNA synthesis by kidney RNA polymerase I (A, left graph) and polymerase II (B, right graph) in the presence and absence of polyamines. The reactions were carried out in standard assay mixture at various substrate concentrations (0.004–0.43 mM UTP) using native DNA (25 μg) as the template and 0.30 and 0.15 unit of kidney polymerase I and II, respectively, in each assay tube. Spermidine (●) and spermine (■) concentrations were as shown in the figure. The results were plotted according to Lineweaver and Burk.

Studies on the effect of various spermine concentrations on the DNA saturation curves were performed with renal RNA polymerase II. The results obtained showed that low spermine concentrations, which stimulated RNA synthesis two- to fourfold, did not change significantly the apparent K_m of polymerase II for native calf-thymus DNA (1.4 μg/ml in the absence of spermine vs. 1.4–1.6 μg/ml in the presence of 0.25–1.0 mM spermine). On the contrary, higher spermine concentrations (2 mM and above) tended to increase the apparent K_m of polymerase II for native DNA, suggesting that the binding of enzyme to DNA could be decreased under these conditions.

The results depicted in Table III show that an almost equal stimulation of RNA synthesis was observed, when spermine was either added 5 min after the start of the reaction, or was present all the time in the reaction mixture. This held true for both kidney RNA polymerases (Table III). These observations suggested that spermine stimulates RNA chain elongation rather than the initiation step of reaction catalyzed by each polymerase. To validate this hypothesis, the effect of polyamines on both elongation and initiation rates were determined using a more detailed series of experiments on these aspects with renal RNA polymerase II.

Average RNA Chain Length. In the presence of 2 mM spermine the average length of the RNA chain formed in the reaction catalyzed by kidney RNA polymerase II was

Table III: Effect of Spermine Added before or 5 min after the Commencement of the Incubation on the Transcription of Saturating Quantities of Native DNA by Pig Kidney RNA Polymerases I and II.[a]

Incubation Conditions	[^{3}H]UMP Incorporated (nmol)	
	Polymerase I	Polymerase II
Control (15 min, 37°)	0.09	0.42
1 mM spermine at 0 time	0.16	1.35
1 mM spermine at 5 min	0.14	1.05

[a] Pig kidney RNA polymerases were incubated in the standard assay system as described under Materials and Methods using 25 μg of native calf-thymus DNA as the template. Spermine was added as indicated in the table. The values are means of triplicate assays.

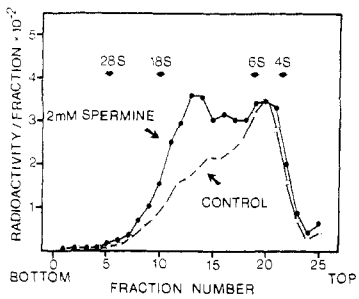

FIGURE 5: Size distribution of RNA synthesized by kidney RNA polymerase II on native calf-thymus DNA in the presence (●) and absence (□) of 2 mM spermine. RNA polymerase reactions were conducted under standard assay conditions with 10 μg of DNA and 0.2 unit of renal RNA polymerase II in each tube. Following incubation, the products formed were isolated and fractionated on 10–30% sucrose gradients as described in the text.

about 2.5-fold longer than that found in the absence of spermine, being 1330 vs. 570 nucleotides, as measured by the UMP/uridine ratio (see Materials and Methods). When an average rate of chain elongation over the 15-min incubation period was calculated from these results, the following figures were obtained: 0.64 nucleotide × sec^{-1} (control) and 1.44 nucleotides × sec^{-1} (2 mM spermine). These rates are of the same order of magnitude as those reported for chick oviduct (Houghton and Cox, 1974) and calf-thymus (Gissinger et al., 1974) RNA polymerases II. Only a slight increase was found to occur in the incorporation of [^{14}C]uridine into the 3′ terminus of the RNA in response to spermine (60 vs. 71 cpm), which suggests an unaltered rate of chain initiation.

Size of the RNA Synthesized in Vitro. The RNA synthesized in vitro by renal RNA polymerase II in the presence of 2 mM spermine contained more high molecular weight RNA when compared to the product formed in the absence of spermine, as judged by 10–30% sucrose density gradient analyses (Figure 5). The major portion of RNA formed in control incubations sedimented at about 6 S, whereas the bulk of the radioactively labeled product formed in the presence of 2 mM spermine sedimented at the 10–18S region. This size difference in the in vitro product further supports the contention that it is the RNA chain elongation that is preferentially stimulated by polyamines during the transcription of native DNA.

Chain Initiation and Elongation Rates. The fact that RNA chain elongation was significantly stimulated with polyamines was proven in experiments, where γ-^{32}P-labeled

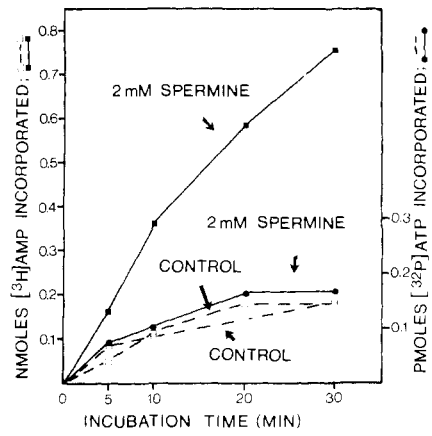

FIGURE 6: Time course of incorporation of [γ-^{32}P]ATP (initiation) and [2,8-^{3}H]AMP (elongation) into RNA in the presence (closed symbols) and absence (open symbols) of 2 mM spermine. Native DNA (10 μg) was used as the template in the reaction containing the standard assay mixture and 0.15 unit of kidney RNA polymerase II. The points are means of triplicate assays. The reaction was terminated at times indicated and processed using the DEAE-cellulose disk technique as described in the text.

ATP was used to measure the rate of chain initiation. The rate of elongation was monitored in parallel with [^{3}H]ATP. Control experiments (see Materials and Methods) showed that under the experimental conditions used, all the [γ-^{32}P]ATP incorporation measured was due to RNA synthesis and not due to phosphorylation of proteins. The results of these studies are summarized in Figure 6, which shows that over the period of 30-min incubation, 2 mM spermine caused only a slight increase in the rate of chain initiation in a reaction catalyzed by renal polymerase II. At the same time, the incorporation of [^{3}H]AMP to RNA was drastically increased, indicating increased rate of chain elongation. The growth of an average RNA chain as calculated from the [^{3}H]AMP/[γ-^{32}P]ATP ratio (Figure 6) was 2.2- to 3.8-fold in favor of spermine over the control at all time intervals.

Inhibition of DNA Transcription by Spermine. As mentioned above, high spermine (5–10 mM) concentrations inhibited both renal RNA polymerases I and II when nonlimiting quantities of native DNA were transcribed (Figure 3), suggesting that the RNA chain elongation process was not the only step of transcription with which polyamines interfered. The inhibitory action of spermine was much more evident, when the amount of DNA template was limiting, as shown in Table IV. Thus, with a shortage of the template, polyamines did not stimulate but inhibited both RNA polymerase I and II activities, possibly owing to a block in the initiation step. This 40–50% inhibition of RNA synthesis caused by 1–2 mM spermine could be overcome by a 5-min preincubation under conditions allowing initiation to take place (not shown). Moreover, these spermine concentrations slightly stimulated RNA synthesis when added after the preincubation.

Effect of Polyamines on the Transcription of Kidney Chromatin. Transcription of kidney chromatin by homologous RNA polymerases was studied using the same conditions as for native DNA transcription. Under these conditions, both polymerases I and II were able to transcribe chromatin with an efficiency ranging from 2 to 10% of that with the same amount of native DNA. Although chromatin preparations contained traces of ribonuclease activity (not shown), the transcription reaction was linear up to 30 min

Table IV: Effect of Spermine on the Transcription of Limiting Quantities of Native DNA by Renal RNA Polymerases I and II.[a]

[Spermine] (mM)	RNA Polymerase I [^{3}H]UMP Incorpd (pmol)	RNA Polymerase I % of Control	RNA Polymerase II [^{3}H]UMP Incorpd (pmol)	RNA Polymerase II % of Control
0	181.0	100	43.0	100
0.25	155.0	86	48.3	112
1.0	128.5	71	28.5	66
2.0	70.6	39	22.8	53
5.0	n.d.[b]		7.4	17

[a] Enzyme assays were carried out under standard reaction conditions using 2 and 0.2 μg of native DNA as templates for RNA polymerases I and II, respectively. The following amounts of enzymes were used: RNA polymerase I, 0.37 unit; RNA polymerase II, 0.1 unit. Values are means of triplicate assays. [b] n.d. = not determined.

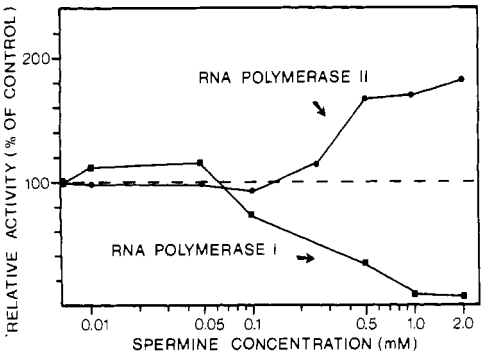

FIGURE 7: Effect of different spermine concentrations on the kidney RNA polymerase I (■) and II (●) activities using chromatin as the template. Enzyme assays were performed in the reaction mixture containing [^{14}C]UTP as the labeled nucleotide (see Materials and Methods) and 62 μg of chromatin DNA/tube. The amounts of RNA polymerases I and II used were 0.12 and 0.14 unit, respectively. The values represent means of triplicate assays and are expressed as percent of controls (= 100, no spermine), which were 10.0 and 14.0 pmol of UMP incorporated for polymerases I and II, respectively.

at 37° in the assay mixture used, pointing to a minimal breakdown of the newly formed RNA. Polyamines did not have any significant effect on the low ribonclease activity present in the chromatin preparations (data not shown). The relatively low ammonium sulfate concentration present in the assay medium allowed only a minimal expression of the endogenous polymerase(s) in the chromatin (results not shown).

The transcription of chromatin by renal RNA polymerase II was stimulated by similar spermine concentrations to that of double-stranded DNA (Figure 7). In contrast, the transcription of chromatin by RNA polymerase I was markedly inhibited even at low spermine concentrations, as illustrated in Figure 7. Thus, polymerase I activity was about 50% inhibited with 0.1–0.25 mM spermine, although a slight tendency to increased activity was evident at lower spermine levels. Similarly, 0.5–1.0 mM spermidine concentration caused about 50% inhibition of chromatin transcription by renal RNA polymerase I. This inhibition of polymerase I probably resulted from a block in the initiation reaction by polyamines, since a short preincubation under conditions allowing initiation to take place was capable of abolishing the inhibition (Table V). In fact, under these conditions an increase in RNA synthesis was seen during the chromatin transcription by renal RNA polymerase I. The optimal spermine concentrations were, however, much lower (0.01–0.1 mM) than those needed for the stimulation of native DNA transcription by the same enzyme. Also, RNA polymerase II activity could be partially inhibited by polyamines, when very limiting template (chromatin) concentrations were used.

Table V: Effect of Various Spermine Concentrations on the Transcription of Kidney Chromatin by RNA Polymerase I When Added after Preincubation.[a]

[Spermine] (mM)	[^{3}H]UMP Incorpd (pmol)	% of Control
0	6.0	100
0.01	7.1	118
0.05	13.7	229
0.1	8.2	137
0.25	5.2	87
0.5	5.2	87
1.0	4.3	72

[a] The reaction was allowed to proceed for 5 min under standard incubation conditions used for chromatin transcription (see Materials and Methods). After this preincubation, the tubes were cooled in ice–water and spermine added in concentrations indicated in the table. The reaction was then run for an additional 15 min at 37°. The amount of RNA polymerase I used was 0.12 unit and that of chromatin 80 μg of DNA/tube. Values are means of triplicate assays.

Discussion

The present study showed that consistent with all eukaryotic RNA polymerases, pig kidney RNA polymerase also occurs in multiple forms. The partially purified RNA polymerase I and II preparations used in this study may represent mixtures of two isozymes, IA/IB and IIA/IIB, respectively, known to be present in some other eukaryotic cells (see Jacob, 1973; Chambon et al., 1974). Indeed, upon further purification, at least pig kidney RNA polymerase I could be resolved into two α-amanitin resistant enzyme fractions, which exhibited almost identical properties (O. Jänne et al., unpublished observations). In agreement with some recent reports on the transcription of chromatin by eukaryotic polymerases (Honjo and Reeder, 1974; Howk et al., 1974) both renal RNA polymerases I and II were shown to be capable of transcribing homologous chromatin.

These studies also demonstrated that the two naturally occurring polyamines, spermidine and spermine, stimulated the transcription by renal RNA polymerases I and II by increasing preferentially the rate of RNA chain elongation. This was confirmed by product analyses such as RNA chain length measurement and RNA size estimation using density gradient centrifugation, as well as by a minimal increase in the [γ-^{32}P]ATP incorporation (initiation) brought about by spermine. The stimulation of chain elongation was not caused by a significant alteration in the apparent K_m of the enzymes with respect to their ribonucleoside triphosphate substrates, but was probably due to interaction of polyamines with the template or enzyme-template complex. Interestingly, the concentrations of polyamines needed to bring about the in vitro stimulation of RNA synthesis were very close to their physiological levels, since the polyamine contents in various mammalian tissues are reported to range from 0.1 to 2 μmol/g of tissue wet weight, the values for rat kidney being about 0.4 and 0.7 μmol/g for spermidine and spermine, respectively (Jänne et al., 1964). It should be pointed out, however, that polyamines may be

compartmentalized within the cell (Raina and Telaranta, 1967), thus making direct comparisons of the in vivo and in vitro concentrations difficult.

The activity of RNA polymerase II could be stimulated when nonlimiting amounts of either DNA or kidney chromatin were used as the template. RNA polymerase I activity was, however, inhibited even at low polyamine concentrations when chromatin was substituted for DNA. This inhibition was caused at the level of initiation, since a preincubation of chromatin with the enzyme and substrates for a brief period prevented the inhibition by polyamines. The reason for the inhibitory effect of polyamines was possibly due to relatively fewer initiation sites available in the total chromatin for RNA polymerase I. Indeed, Honjo and Reeder (1974) have shown that in *Xenopus* chromatin the number of binding sites for homologous RNA polymerase I was three times less than those available for polymerase II. The chromatin preparation we used in these studies seemed also to have much fewer binding sites for RNA polymerase I than for polymerase II (O. Jänne et al., unpublished observations). The importance of the number of available initiation sites as a modulator of the spermine action was imminent for RNA polymerase II as well, since its activity too could be inhibited by spermine when chromatin was used in limiting quantities. Moreover, both RNA polymerases I and II were inhibited by polyamines when limiting quantities of native DNA were employed as the template. It can thus be concluded that inhibition of RNA synthesis by polyamines occurs at the initiation step and that RNA polymerase I is more sensitive to this inhibition than RNA polymerase II.

The mechanism by which polyamines at physiological concentrations and at nonlimiting quantities of templates stimulate the elongation reaction could be due to a prolonged retention of the transcription complex comprising DNA, RNA polymerase, and nascent RNA. This will thus prevent premature release of the newly formed RNA and thus account for a production of longer RNA chains in the presence of polyamines. Herbst et al. (1973) have offered a similar explanation for the effect of spermine on *E. coli* RNA polymerase activity. Spermidine and spermine have also been reported to increase the activity of *E. coli* RNA polymerase at least partly by counteracting the inhibition caused by the RNA product (Abraham, 1968). Furthermore, spermidine has been reported to promote asymmetric transcription of DNA by bacterial (*Micrococcus lysoleikticus*) RNA polymerase (Gumport, 1970). It is not known whether such reactions play any role in promoting elongation of RNA chains catalyzed by mammalian RNA polymerases.

The dual function of spermine and perhaps of other polyamines as well, i.e. the stimulation of RNA chain elongation at physiological or low concentrations and inhibition of chain initiation at higher concentrations, makes these basic aliphatic amines attractive intracellular regulators of transcription. It is not known, however, whether polyamines specifically promote the elongation of only some RNA transcripts or stimulate the elongation of all the RNA molecules. An early increase in the levels of polyamines during growth or development and in connection with hormone action could produce longer RNA molecules in vivo which may be required for their proper processing. Thus, a minimal length of ribosomal RNA may be necessary for its methylation by specific methylases. Similarly, specificity of polyadenylation of messenger RNA by poly(A) polymerases may lie in the length of the messenger RNA used as the primer. It is well recognized that the length of primers is crucial for poly(A) polymerase activity (Winters and Edmonds, 1973; Rose et al., 1975). Finally, it is possible that very high levels of polyamines could control the amount and length of RNA produced by inhibiting the initiation process.

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