HeLa Cell Deoxyribonucleic Acid Dependent RNA Polymerases: Function and Properties of the Class III Enzymes†

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ABSTRACT: The class III DNA dependent RNA polymerases (nucleoside triphosphate:RNA nucleotidyltransferase EC 2.7.7.6) from HeLa cells have been solubilized and characterized as to function and properties. Two chromatographically distinct forms of enzyme III, designated polymerases III_A and III_B, can be resolved when cell extracts are chromatographed on DEAE-Sephadex columns. Enzymes III_A and III_B exhibit nearly identical catalytic properties such as divalent cation stimulation, broad biphasic ammonium sulfate optima, and characteristic α-amanitin sensitivities which clearly distinguish them from the homologous enzymes, forms I and II. Polymerases III_A and III_B are both primarily localized in the nucleus (>60%). The most notable characteristic of the class III enzymes is a unique sensitivity to inhibition by α-amanitin (50% inhibition at 15 μg/ml). HeLa cell enzyme I is not inhibited by the mushroom toxin even at very high concentrations (>400 μg/ml), while HeLa cell polymerase II is inhibited by very low concentrations of amanitin (50% inhibition at 0.003 μg/ml). The three major classes of enzyme (I, II, III) exhibit characteristic sensitivities to α-amanitin whether assayed in nuclei, crude homogenates, or in a chromatographically purified state. Using a nuclear in vitro RNA synthesizing system to investigate the α-amanitin sensitivities of the synthesis of tRNA precursor (4.5S pre-tRNA) and 5S ribosomal RNA, it was found that the synthesis of these RNA species was inhibited 50% at 15 μg/ml of α-amanitin. The α-amanitin inhibition curves for the synthesis of pre-tRNA–5S ribosomal RNA in nuclei and the α-amanitin titration curves for the partially purified class III enzymes (III_A and III_B) are identical. These data, therefore, show that the in vivo functional role of the class III RNA polymerases (III_A–III_B) is the transcription of the genes coding for transfer RNA and 5S ribosomal RNA.

The multiple forms of DNA dependent RNA polymerases, I, II, and III (Roeder and Rutter, 1969), and especially forms I and II, have been purified and characterized in a number of mammalian systems (for review see Watson, 1970; Chambon et al., 1974). Nucleolar RNA polymerase I (Roeder and Rutter, 1970a) which is not inhibited by α-amanitin (Kedinger et al., 1970; Lindell et al., 1970) is responsible for the synthesis in vivo of precursor to ribosomal 18S and 28S RNA (Blatti et al., 1970; Reeder and Roeder, 1972). RNA polymerase II located in the nucleoplasm (Roeder and Rutter, 1970a) is sensitive to inhibition by low levels of α-amanitin and is presumably responsible for the synthesis of messenger RNA (Blatti et al., 1970; Zylber and Penman, 1971). But in past years, except for a few cases (Roeder and Rutter, 1970a; Lindell et al., 1970; Seifart et al., 1972; Sergeant and Krsmanovic, 1973), enzyme form III was not isolated from mammalian cells (Kedinger et al., 1970; Watson, 1970; Sugden and Keller, 1973; Hall and Smuckler, 1974; and others). The existence and function in mammalian cells of this enzyme form, present in many lower eukaryotic organisms (Roeder and Rutter, 1970b; Adman et al., 1972; Ponta et al., 1972; Roeder, 1974; Young and Whiteley, 1975), have, therefore, been under question.

Recently, RNA polymerase III has been described in a number of mammalian cell types. Two forms of enzyme III, designated III_A and III_B, were reported to be present in mouse myeloma cells (Schwartz et al., 1974; Weinmann and Roeder, 1974a,b) and in KB cells (Weinmann et al., 1974, 1975). However, only one form of enzyme III was described in Chinese hamster kidney cells by Austoker et al. (1974), in HeLa cells by Seifart and Benecke (1975), and in calf thymus by Weil et al. (1974) and Weil and Blatti (1975). We report here the presence of two forms of enzyme III (III_A and III_B) in HeLa cells. The reasons for the lack of multiple forms of enzyme III (III_A and III_B) in some mammalian cell types are unknown, but all forms of enzyme III described exhibit distinctive properties: elution after polymerase II on DEAE-Sephadex columns, broad ammonium sulfate optima, specific divalent cation optima, and a unique α-amanitin sensitivity (50% inhibition at 10–30 μg/ml).

The nuclear–cytoplasmic localization of enzymes III_A and III_B has previously been investigated by Schwartz et al. (1974). Their results indicate that enzyme III_A was primarily found in the nucleus, while enzyme III_B was found only in the cytoplasm of mouse myeloma cells. In contrast to these results, we report here that both forms of enzyme III (III_A and III_B) are found in HeLa cell nuclei.

The three major classes of HeLa cell RNA polymerases I, II, and III exhibit characteristic sensitivities to α-amanitin inhibition whether assayed in crude cellular homogenates, in a partially purified state, or in nuclei (see below). Therefore, using the well characterized, nuclear in vitro

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HeLa cell RNA synthesizing system (Zylber and Penman, 1971; McReynolds and Penman, 1974a), the synthesis of a particular species of RNA can be titrated with α-amanitin. The concentration of α-amanitin required for 50% inhibition of synthesis will be characteristic of the polymerase form transcribing the corresponding genes (in the HeLa system: polymerase I, not inhibited at >400 μg/ml; polymerase II, 50% inhibition at 0.003 μg/ml; polymerase III (III_A-III_B) 50% inhibition at 15 μg/ml).

We have used this approach in the HeLa system to titrate the synthesis of pre-tRNA and 5S ribosomal RNA in vitro in nuclei with α-amanitin (preliminary report: Weil et al., 1974). The inhibition curve for the synthesis of these RNA species is identical with that obtained for the solubilized class III enzymes (III_A-III_B), with 50% inhibition of synthesis occurring at 15 μg/ml. These data, therefore, show that the HeLa cell class III enzymes (III_A-III_B) are responsible for the transcription of pre-tRNA and 5S ribosomal RNA in vivo.

The results of these experiments independently demonstrate and are in excellent agreement with those obtained in the mouse myeloma system by Weinmann and Roeder (1974a,b). These workers, using the technique of α-amanitin titration of nuclear RNA synthesis, showed that the myeloma class III enzymes (III_A-III_B) are responsible for the synthesis of pre-tRNA and 5S RNA. Weinmann et al. (1974, 1975) also showed that, in Adenovirus-2 infected KB cells, the class III host enzymes (III_A-III_B) are responsible for the synthesis of Adenovirus-2 specific 5.5S RNA (Price and Penman, 1972) and host cell 5S RNA. Since the properties of the two enzyme forms III_A-III_B are so similar, it is at present not possible to distinguish which form(s) is responsible for the synthesis of these two low-molecular-weight RNAs. However, the results of these experiments in different mammalian cell types clearly prove that the enzyme III system (III_A-III_B) is responsible for the synthesis of pre-tRNA and 5S RNA, the third major class of cellular RNAs.

Materials and Methods

Biochemicals. All chemicals were reagent grade. Tritium-labeled UTP (specific activity >20 Ci/mmol), α-[^{32}P]GTP (specific activity 10-50 Ci/mmol), and ^{32}P-labeled H_3PO_4 carrier free, were obtained from New England Nuclear. α-Amanitin was purchased from Calbiochem, calf thymus DNA (grade I) was from Sigma Chemical Company, and poly[d(A-T)] was obtained from both Miles Laboratories and PL Biochemicals. Crystalline bovine serum albumin was purchased from Pentex-Miles. Tissue culture media (DME[1]) was from Gibco. Antibiotics were from Calbiochem. NP-40 was from Shell Chemical Company. Yeast carrier RNA was from Miles Laboratories.

Cells. HeLa cells were grown in glass roller bottles or plastic tissue culture plates as monolayers in DME (Dulbecco's Modified Basal Medium) supplemented with 12.5% horse serum and 2.5% fetal calf serum in the presence of ampicillin (25 μg/ml), penicillin (121 μg/ml), and streptomycin (34 μg/ml). Original stocks of HeLa cells were kindly provided by Dr. D. Sirbasku.

Solutions. All buffers and media were prepared from reverse-osmosis purified deionized water. When present, dithiothreitol was added immediately before use.

[1] Abbreviations used: DME, Dulbecco's Modified Basal Medium.

Buffer A is 50 mM Tris-HCl (pH 7.9 adjusted at 25 °C), 33% glycerol, 5 mM $MgCl_2$, 0.1 mM Na_2EDTA, and 0.5 mM dithiothreitol. Buffer B was phosphate buffered saline (from Gibco). Buffer C is 10 mM Tris-HCl (pH 7.9), 6 mM KCl, 1.6 mM $MgCl_2$, and 1 mM dithiothreitol. Buffer D is 17 mM Tris-HCl (pH 7.9), 17% glycerol, 10 mM KCl, 3.3 mM $MgCl_2$, 83 mM ammonium sulfate, and 0.83 mM dithiothreitol. Buffer E is the same as buffer C, except that the concentration of dithiothreitol is 2 mM, and 6 mM NaCl replaces the 6 mM KCl. Buffer F is 10 mM Tris-HCl (pH 7.9), 25% glycerol, 0.1% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, and 2 mM dithiothreitol. Buffer G is 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, and 0.5% sodium dodecyl sulfate. Buffer H is 50 mM Tris-HCl (pH 7.9), 250 mM sucrose, 25 mM KCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol. Buffer I is 340 mM sucrose, 15 M $MgCl_2$.

Solutions containing ammonium sulfate were prepared by the addition of the appropriate amount of a concentrated solution of ammonium sulfate, 4 M, pH 7.9 (adjusted at 25 °C with NH_4OH).

Conductivity Measurements. The ammonium sulfate concentration of a given sample was determined by taking a 10-μl aliquot, mixing with 1 ml of water, and measuring the conductivity using a Radiometer CDM2b conductivity meter. The concentration of the sample was then found by extrapolation from a standard curve previously constructed using ammonium sulfate solutions of known concentration.

Protein, DNA, and Cell Number Determinations. Protein concentrations were estimated by 280:260 absorption ratios or measured by the method of Lowry et al. (1951) using bovine serum albumin as the protein standard. Samples were precipitated with trichloroacetic acid before Lowry determinations. DNA measurements were made by the method of Burton (1956). Cell number determinations were made utilizing a Coulter Counter.

Preparation of Ion-Exchange Resins and Chromatography. DEAE-Sephadex A-25 was obtained from Pharmacia. The resin was suspended in 4 volumes of reverse-osmosis purified water and titrated to pH 7.9 with 2 M Tris base. The exchanger was then washed with 8 volumes of 0.5 M ammonium sulfate, pH 7.9, followed by 16 volumes of water. The resin was equilibrated with 8 volumes of buffer A, 50 mM in ammonium sulfate, and resuspended in buffer A, 50 mM in ammonium sulfate and made 0.03% in toluene as a 1:1 (buffer-resin) slurry, and stored at 4 °C until use.

DE-52 was obtained from Whatman Inc. The resin was suspended in 8 volumes of ten-times concentrated buffer A (500 mM Tris-HCl, pH 7.9) and allowed to equilibrate overnight. The resin was resuspended two times in 4 volumes of buffer A, 50 mM in ammonium sulfate, and finally resuspended in the same solution, made 0.03% in toluene as a 2:1 (resin-buffer) slurry, and stored at 4 °C until use.

All resin slurries were warmed to room temperature and degassed by vacuum before use. Columns were poured, placed at 4 °C, and equilibrated with 4 volumes of cold buffer A. Samples adjusted to 50-60 mM ammonium sulfate in buffer A were then applied to the column at a rate of 1 column volume per hour. Columns were washed with 2 column volumes of buffer A, 50 mM in ammonium sulfate, and eluted using a linear ammonium sulfate gradient of 5 column volumes (50-500 mM) in buffer A at a flow rate of 1.5 column volumes per hour. Fractions of 2-3% of the total gradient volume were collected. The amount of protein applied to DEAE columns was about 2 mg per ml of bed vol-

ume.

Cell Fractionation Procedures. Two methods of cellular fractionation were used to localize polymerase III activity in subcellular fractions. Whenever cells were harvested, the monolayers were first washed once with cold buffer B, then harvested by scraping the cells into cold buffer B, collected by low-speed centrifugation, resuspended in the same solution, and repelleted. The cell pellet was subsequently resuspended in the appropriate buffer.

Method 1. The cell pellet was resuspended in 6 volumes of buffer H and homogenized by hand in a tight-fitting, Teflon–glass homogenizer with 15–20 passes of the pestle. Completeness of cell breakage was monitored by microscopy. Nuclei were sedimented by centrifugation at 4500*g* for 10 min. The supernatant (cytoplasmic fraction) was removed and saved. The pellet was resuspended in a small volume of buffer H and re-sedimented. The supernatant was removed and combined with the previous one. Nuclei were resuspended in a volume of buffer A equivalent to 1 volume per 10^8 cells for subsequent solubilization of RNA polymerase (see below). The combined cytoplasmic fractions were centrifuged at 10 000*g* for 15 min. The supernatant was made 0.1 mM in EDTA, 33% in glycerol, and 50 mM in ammonium sulfate and chromatographed on DEAE-Sephadex.

Method 2. Nuclei were isolated from the cell pellet by a modification of the method described by Schwartz et al. (1974). Briefly, cells were homogenized in buffer I in a hand-driven, Teflon pestle homogenizer and nuclei pelleted by low-speed centrifugation. The supernatant (cytoplasm) was adjusted to 50 mM Tris-HCl (pH 7.9), 33% glycerol, 0.5 mM dithiothreitol, and 50 mM in ammonium sulfate and chromatographed in DEAE-Sephadex. The nuclear pellet was resuspended in 1 volume of buffer I per 10^8 cells homogenized and diluted with 4 volumes of 2.3 M sucrose. Nuclei were then pelleted by centrifugation (27 000 rpm for 1 h, SW 27 rotor). Nuclei were resuspended in buffer A for enzyme solubilization as described below.

RNA Polymerase Solubilization. RNA polymerases were solubilized by the method of Schwartz et al. (1974). RNA polymerase from whole cells or nuclei were solubilized by the following procedure. All steps were performed at 0 °C. Cells or nuclei in one volume of buffer A per 10^8 cells were made 0.3 M in ammonium sulfate by the addition of the appropriate amount of a 4 M stock solution. The resulting viscous solutions were sonicated with a Branson sonicator using the large probe on the high setting for a total of 2 min in 10-s bursts. When the suspension readily formed drops at the end of a Pasteur pipet, sonication was complete. The suspensions were centrifuged for 50 min at 50 000 rpm in a Ti50 Spinco rotor. The supernatant, designated F1, was diluted to 0.1 M ammonium sulfate by the addition of buffer A and recentrifuged for 50 min at 50 000 rpm (Ti50 rotor). The supernatant, designated F2, was diluted to 0.05 M ammonium sulfate by the addition of buffer A and recentrifuged for 110 min at 35 000 rpm (Type 35 Spinco rotor). The supernatant F3 was then applied to DEAE ion-exchange resins for fractionation of the individual polymerase forms. After the above centrifugations, the pellets which consisted of aggregated chromatin and cellular debris were discarded.

RNA Polymerase Assay. Assays were performed essentially as described previously (Weil and Blatti, 1975). The assays were done in a final volume of 60 μl with the following components initially present in each assay tube in a volume of 35 μl: 3 μmol of Tris-HCl (pH 7.9), 100 nmol of $MnCl_2$, 60 nmol of dithiothreitol, either 20 μg of calf thymus DNA or 3 μg of poly[d(A-T)] as template, 36 nmol each of GTP, CTP, and ATP, 600 pmol of UTP and 0.5 μCi of [^{3}H]UTP. When present, α-amanitin was added to give the proper final concentration. Depending upon the experiment, between 10 and 25 μl of enzyme solution was added. The remaining volume was made up with buffer A. The reaction was initiated by enzyme addition and incubated at 37 °C for 20 min. Termination of the reaction occurred by taking 50-μl aliquots from each tube and spotting on DE-81 filter discs. Filter discs were processed to determine [^{3}H]UMP incorporation as described (Weil and Blatti, 1975). One unit of activity represents the incorporation of 1 pmol of UMP into RNA in 20 min under the above set of conditions.

Synthesis of RNA in Nuclei. RNA was synthesized in HeLa cell nuclei in vitro by a slight modification of the method of McReynolds and Penman (1974a). Cells were harvested as described above. The cell pellet was resuspended in 10 volumes of buffer C and incubated for 10 min at 0 °C to swell the cells. NP-40 was added to 0.5%, and the cells were disrupted by rapid agitation on a Vortex mixer (1–1.5 min, 15-s bursts). Nuclei were collected by low-speed centrifugation, washed once in buffer C, and recentrifuged. Nuclei were resuspended in 4 volumes of buffer D and used for the synthesis of RNA in the following system. RNA was synthesized in a final volume of 500 μl containing the following added components at these final concentrations: 100–200 μCi/ml of [^{3}H]UTP, 100 μM ATP, CTP, and GTP, and 5 μM UTP; *or* 100–200 μCi/ml of α-[^{32}P]GTP, 100 μM ATP, CTP, and UTP and 5 μM GTP (depending upon the radioactive nucleotide used). When present α-amanitin was added to the substrate solution to give the proper final concentration. Reactions were initiated by the addition of 300 μl of nuclei and incubated at 26 °C for 20 min. Synthesis was terminated by the addition of 1.5 ml of prechilled buffer C (0 °C). Nuclei were pelleted by centrifugation at 2000*g* for 15 min. The supernatant was drawn off, made 0.5% in sodium dodecyl sulfate, and phenol extracted two times at room temperature. To the final aqueous phase was added yeast carrier RNA to 10 μg/ml. RNA was precipitated by the addition of 2.5 volumes of cold absolute ethanol and storage overnight at −20 °C. The radioactive RNA was recovered by centrifugation, lyophilized to dryness, and redissolved in a minimum of the appropriate buffer depending upon the final use of the labeled RNAs. The volume of the reaction mixture was varied depending upon the experiment, but never exceeded 500 μl. When analysis of specific species of RNAs for an α-amanitin titration was performed, a constant amount of labeled tRNA was added to each reaction mixture before initiation of the reaction to determine recovery and to monitor ribonuclease action.

Analysis of in Vitro RNA. RNA synthesized in vitro was analyzed either on 10–30% sucrose gradients in buffer G or on 9 cm, 10% acrylamide gels as described by Maizel (1969). The sucrose gradients were collected by puncturing the bottom of the tubes and collecting fractions. Aliquots of each fraction were spotted on DE-81 filter discs and processed as described above for assay discs. The gels were fractionated on a Gilson gel slicer. The crushed slices were made up to 1 ml with water and counted after the addition of 10 ml of scintillation fluid (66% Omnifluor–33% Triton X-100).

Table I: Purification of HeLa Cell RNA Polymerases.[a]

Purification Step	Total Activity (Units)	Protein (mg)	Specific Activity (Units/μg of Protein)
F1	69 500	550	0.13
F2	130 200	447	0.30
F3	185 800	372	0.50
DEAE-Sephadex	191 400	104	1.84
Polymerase I	132 600	47	2.82
Polymerase II	52 500	33	1.59
Polymerase III_A	2 700	16	0.17
Polymerase III_B	3 500	8	0.44

[a] All fractions (F1, F2, F3) were assayed at 50 mM ammonium sulfate. Fractions F1, F2, and F3 are those described in the purification procedure section of Materials and Methods. DEAE-Sephadex refers to all the RNA polymerase containing fractions on a DEAE-Sephadex column. Polymerase I, II, III_A, and III_B refer to the appropriate enzyme containing fractions from the corresponding DEAE-Sephadex column. DEAE-Sephadex columns were assayed as described in the legend to Figure 1. The above figures were obtained from one representative experiment. RNA polymerases were purified from 6.8 × 10^9 HeLa cells.

Preparation of Marker RNA. Radioactive marker RNAs were prepared from rat liver as described previously (Weil and Hampel, 1973). ^{32}P-Labeled RNA's were prepared from HeLa cells grown exponentially in carrier-free [^{32}P]NaH_2PO_4 (final concentration, 20 μCi/ml). The cells were grown to confluency (two generations) and harvested as described above. The cells were resuspended in buffer E, swollen for 10 min at 0 °C, and broken by homogenization with a Teflon–glass homogenizer. A cytoplasmic fraction was obtained by centrifugation at 10 000*g* for 15 min. Sodium dodecyl sulfate was added to 0.5%, and the cytoplasmic RNAs were phenol extracted as described above.

Radioactive RNAs obtained were fractionated on 10–30% sucrose gradients in buffer G, the appropriate regions of the gradients were pooled to obtain 4–7S, 18S, and 28S RNAs, and each RNA species was recovered by ethanol precipitation.

Processing of Pre-tRNA. Two methods of processing of the pre-tRNAs synthesized in HeLa nuclei were used. The first method (Bernhardt-Mowshowitz, 1970) which was used consisted of incubating the pre-tRNA with a postribosomal supernatant under high pH and high salt conditions. The second method used was that described by Marzluff et al. (1974). In this case the pre-tRNAs were incubated with a detergent wash (buffer F) of nuclei. In both cases harvested cell pellets were resuspended in hypotonic buffer E and swollen for 10 min at 0 °C. The swollen cells were broken by homogenization in a glass–Teflon homogenizer and treated as described by Bernhardt-Mowshowitz (1970) or Marzluff et al. (1974) to produce a crude preparation of processing enzymes. In vitro low-molecular-weight RNAs were processed at 37 °C in the presence of added marker tRNA. Processing was terminated by the addition of 1 volume of 0.05 M NaCl, 0.1 mM EDTA, and sodium dodecyl sulfate to 1%. RNA was then phenol extracted, ethanol precipitated, and analyzed on 10% polyacrylamide gels as described above.

Results

Purification, Chromatographic Properties, and Amounts of HeLa Cell RNA Polymerases. HeLa cell RNA polymerases can be efficiently solubilized using the high salt, sonication procedure. As Table I illustrates, total solubilized activity increases throughout the purification procedure (F1 to F3) as cellular debris and aggregated chromatin are removed by dilution and high-speed centrifugation. This increase in activity is probably the result of removal of nonspecific inhibitors and endogenous HeLa DNA which is more highly double stranded and not as efficient a template as the calf thymus DNA used here to measure activity (Ferencz and Seifart, 1975; Gissinger et al., 1974).

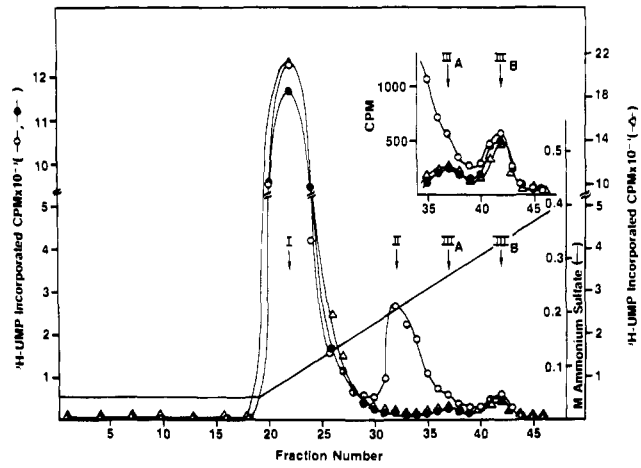

FIGURE 1: DEAE-Sephadex chromatography of a whole cell extract. Fraction F3 (derived from 1.9 × 10^9 cells as described in the text), containing 108 mg of protein, was applied to a pre-equilibrated DEAE-Sephadex column 1.5 × 30 cm. The column was washed with 150 ml of buffer A, 0.05 M in ammonium sulfate, collecting 14-ml fractions (fractions 1–16). Polymerases were eluted with a 200-ml linear ammonium sulfate gradient, 0.05–0.5 M, collecting 6-ml fractions (17–46). Twenty-five-microliter aliquots of fractions were assayed with calf thymus DNA, either in the absence (○) or presence (●) of 0.5 μg/ml of α-amanitin *or* with poly[d(A-T)] as template (△) in the presence of 0.5 μg/ml of α-amanitin. The final salt concentrations in the activity assays were not adjusted to the respective optimum for each of the enzymes but were determined by the 5/12 dilution of salt present in the fractions during the RNA polymerase activity assays. (See Methods section under RNA Polymerase Assay). Ammonium sulfate concentration is indicated by the solid line (—).

Fraction F3 was applied to either DEAE-Sephadex or DEAE-cellulose columns. Figure 1 illustrates a typical elution profile of a whole cell extract chromatographed on DEAE-Sephadex. Four peaks of activity are evident when the column fractions are assayed with calf thymus DNA as template in the presence and absence of low levels (0.5 μg/ml) of α-amanitin. In order of elution these activity peaks are designated polymerases: I, II, III_A, and III_B. These results are in agreement with the work done by Schwartz et al. (1974) in the mouse myeloma system. These workers described four forms of RNA polymerase activity when whole cell extracts were chromatographed on DEAE-Sephadex.

All of these enzyme forms exhibit distinctive catalytic properties (see below) and, when rechromatographed on DEAE-Sephadex columns, each enzyme form elutes at its characteristic ammonium sulfate concentration: polymerase I at 0.09 M, polymerase II at 0.2 M, polymerase III_A at 0.22–0.28 M, and polymerase III_B at 0.32–0.38 M. Recovery of activity from these rechromatography experiments was always >75% (data not shown). The exact elution positions of polymerases III_A and III_B are the most variable, but polymerase III_A always elutes with, or at a slightly higher ammonium sulfate concentration than, enzyme II and, thus, is always contaminated with polymerase II when only purified using DEAE-Sephadex chromatography. Form III_B always elutes at an ammonium sulfate concen-

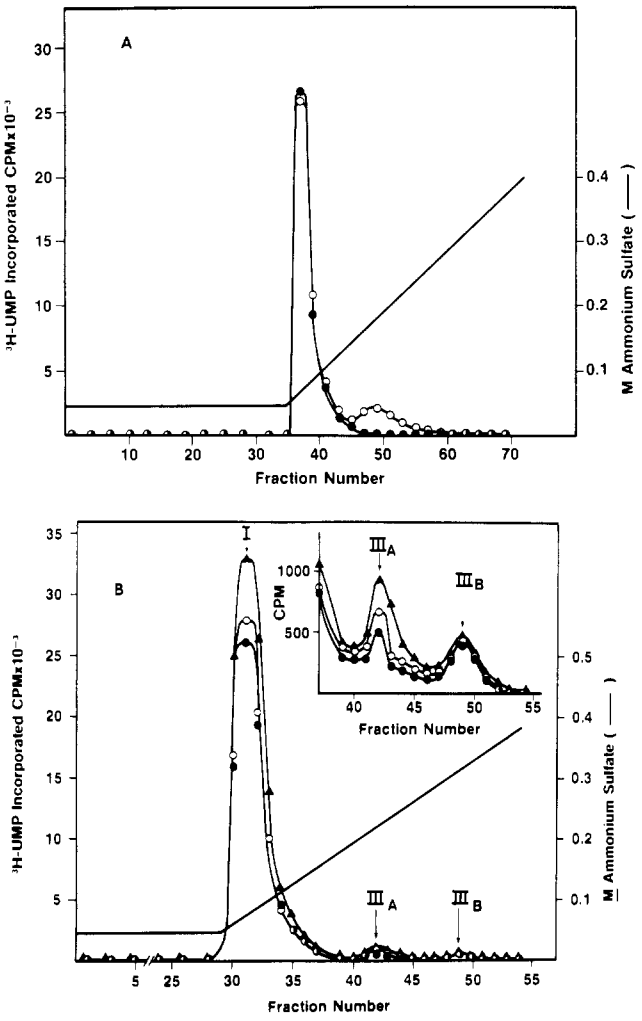

FIGURE 2: Chromatographic properties of HeLa cell class III enzymes on DEAE ion exchangers. In (A) fraction F3, derived from 3.1×10^9 cells (containing 510 mg of protein), was applied to a DEAE-cellulose column (2.5 × 27 cm). The column was washed as described and polymerase eluted with a 600-ml linear ammonium sulfate gradient (0.05–0.5 M) collecting 17-ml fractions. Aliquots of each fraction (25 μl) were assayed for enzyme activity with calf thymus DNA as template. Fractions 36–42 from the DEAE-cellulose column in (A) were pooled and diluted to 0.05 M ammonium sulfate. The pooled fractions containing 90 mg of protein were applied to a 2.5 × 16 cm DEAE-Sephadex column depicted in (B). The column was washed with 150 ml of buffer A, 0.05 M in ammonium sulfate, and eluted with a linear gradient (425 ml), 0.05–0.5 M ammonium sulfate. Fractions of 12 ml were collected. Twenty-five-microliter aliquots of fractions were assayed. Activity with calf thymus DNA as template was determined in the absence (○) or presence (●) of 0.5 μg/ml of α-amanitin; activity with poly[d(A-T)] as template (▲). Ammonium sulfate concentration is indicated by the solid line.

tration higher than III_A and is completely separated from the latter.

In contrast to the elution profile of polymerase activities when cellular extracts are chromatographed on DEAE-Sephadex, DEAE-cellulose chromatography reveals only two forms of RNA polymerase activity. Figure 2A illustrates a typical DEAE-cellulose chromatogram of a whole cell extract. Peaks one and two correspond to the α-amanitin insensitive and sensitive polymerases I and II described in HeLa cells by Sugden and Keller (1973) and in other mammalian systems (Watson, 1970). However, when the polymerase I activity peak is pooled, diluted to 0.05 M ammonium sulfate, and rechromatographed on DEAE-Sephadex,

Table II: Stimulation of Enzyme Activity by Poly[d(A-T)] Template.[a,b]

	Form I cpm	Form II cpm	Form III_A cpm	Form III_B cpm
Calf thymus DNA template	16 600	3 400	2800	2500
Poly[d(A-T)] template	30 500	18 000	5600	6200
Fold stimulation	1.8	5.3	2.0	2.5

[a] Enzymes were assayed at their respective salt optima and cpm incorporated listed above represent maxima with a given template. All assays were performed as described in the Materials and Methods section of the text. Sources of enzyme fractions were as described in the text. [b] The size of the poly[d(A-T)] used as template as determined by neutral sucrose gradient centrifugation was 9.5S.

Table III: RNA Polymerase Levels in HeLa Cells.[a]

Units of Enzyme per 10^8 Cells[b]		
Polymerase I	Polymerase II	Polymerase III[c]
2300	700	100

[a] Units of enzyme were determined from RNA polymerase profiles on DEAE-Sephadex columns. All values are averages calculated from six separate experiments. [b] When enzymatic activity is assayed under the conditions of Roeder (1974), the values of units/10^8 cells are approximately two times greater. [c] Polymerase III above represents the sum of III_A and III_B activities.

three peaks of enzyme activity are evident. Figure 2B shows that the DEAE-cellulose peak one was heterogenous, actually containing enzymes I, III_A, and III_B. This anomalous chromatographic behavior of enzyme III (III_A–III_B) has been reported previously for KB cell enzyme III (Sergeant and Krsmanovic, 1973) and more recently by Austoker et al. (1974) and Seifart and Benecke (1975). The peak of polymerase III_A in Figure 2B is partially sensitive to inhibition by α-amanitin (0.5 μg/ml) but this is due to the inclusion of a small amount of enzyme II when peak one from DEAE-cellulose was pooled. When α-amanitin resistant fractions from DEAE-cellulose are carefully pooled to exclude enzyme II, the subsequently obtained DEAE-Sephadex polymerase III_A is totally resistant to low levels of α-amanitin (data not shown). For the characterization studies of enzymes III_A and III_B described below, these polymerases were therefore partially purified by combined DEAE-cellulose and DEAE-Sephadex chromatography as outlined above.

The columns illustrated in Figures 1 and 2B have been assayed using either calf thymus DNA or poly[d(A-T)] as template. The increase in activity when poly[d(A-T)] is used as template is small and variable under the conditions used to assay these columns. This is because of the variable salt elution position of the form III enzymes. Using poly[d(A-T)] as template, the salt optima for all polymerases are very sharp, with a maximum of 50 mM ammonium sulfate (see below). Table II illustrates the increase in activity observed when using poly[d(A-T)] as template. Note that for the class III enzymes the increase in activity is about twofold. A similar fold increase in activity was shown for the class III enzyme from calf thymus (Weil and Blatti, 1975). These data are in contrast to that obtained by Roeder and co-workers (Schwartz et al., 1974; Jaehning et al.,

Table IV: Distribution of RNA Polymerases in Nuclear and Cytoplasmic Subfractions of HeLa Cells.[a]

Method[b]	Subcellular Fraction	Pol I[c]	Pol II	Pol III_A	Pol III_B
	Nuclei	65.4	73.6	69.7	46.2
1	Cytoplasm	1.9	4.8	17.9	27.7
	N + C	67.3	78.4	87.6	73.8
	Nuclei	69.7	70.9	63.6	25.6
2	Cytoplasm	0.2	0.7	1.1	1.6
	N + C	69.9	71.6	64.8	27.2

[a] HeLa cells were grown and harvested as described above. About 30 ml of packed cells was obtained. The cells were divided into three equal portions. One portion of whole cells was used directly for isolation of enzymes. The other two portions were fractionated to nuclei and cytoplasm by either Method 1 or 2 and enzymes were isolated from each of the subcellular fractions. Enzymes were solubilized and chromatographed on DEAE-Sephadex as described above. The amounts of polymerases I, II, III_A, and III_B in whole cells as determined from DEAE-Sephadex chromatography are respectively: 101 000; 30 400; 15 300; 9000 units. [b] Methods 1 and 2 refer to the two different procedures for fractionation of cells to nuclei and cytoplasm as described above. Method 2 is a modification of the procedure described by Schwartz et al. (1974). The amounts of the various enzymes in the nuclear fractions were corrected for recovery of nuclear DNA (Method 1: 84.5% recovery; Method 2: 63.3% recovery). [c] The values listed refer to percent of activity found in whole cells.

1975; Weinmann et al., 1975) who report a five- to tenfold increase in activity for the class III enzymes when poly[d(A-T)] is used as template. Although the exact reasons for these differences are unknown, significant amounts of polymerases III_A and III_B can still be assayed in HeLa cells with native calf thymus DNA. Table III shows the levels of the three major polymerase forms present in HeLa cells. The units of enzymes were determined from DEAE-Sephadex elution profiles and represent activity assayed with calf thymus DNA as template.

Nuclear–Cytoplasmic Localization of HeLa RNA Polymerases. HeLa cells were fractionated into nuclei and cytoplasm by two different procedures as described in Methods. Glycerol was added to a final concentration of 33% to the cytoplasmic fractions immediately after nuclei separation to stabilize the enzymes in the cytoplasmic fractions against inactivation. However, despite this precaution, approximately 30% of the total activity (Polymerases I, II, III) is lost, presumably by inactivation. From Table IV it can be seen that, while there are polymerases III_A and III_B in the cytoplasmic subfractions of HeLa cells, >63% of the total activity of both forms of enzyme III is found in the nuclei of these cells. Also it can be seen that the amount of each enzyme form which leaches out of the nuclei is different ($III_B > III_A > II > I$). Thus, enzymes III_A and III_B have a greater tendency to leach out of nuclei. Since the III enzymes transcribe the comparatively small tRNA and 5S RNA genes, these enzymes should transverse their transcription cycle in a relatively shorter time, allowing selective leakage of these enzymes during subcellular fractionation.

Catalytic Properties of HeLa Cell RNA Polymerases. Enzymes I and II used for the experiments described below were obtained from DEAE-Sephadex columns. Enzymes III_A and III_B were obtained as described above from DEAE-cellulose and DEAE-Sephadex columns. Before use, the polymerase containing fractions were pooled, diluted, and adsorbed to small DEAE-Sephadex columns and concentrated by batch elution with the appropriate concentration of ammonium sulfate. Recovery of activity using this concentration procedure always approached 100% for all four enzyme forms.

The ammonium sulfate activation curves for the four forms of HeLa RNA polymerases were determined with both poly[d(A-T)] and calf thymus DNA as template. All enzymes exhibit similar monophasic salt curves with poly[d(A-T)] as template (optimum about 50 mM ammonium sulfate, not shown). When the salt titration curves of these enzymes are determined with calf DNA as template, they all exhibit distinctive properties. Polymerases I and II give monophasic salt titration curves with optima at 40 and 100 mM ammonium sulfate, respectively (not shown). On the other hand, polymerases III_A and III_B display broad, biphasic salt activation curves. III_A has optima at 45 and 160 mM ammonium sulfate, while III_B has optima at about 60 mM and 150–200 mM ammonium sulfate (Figure 3). Broad monophasic curves for polymerase III have been described in Chinese hamster kidney cells (Austoker et al., 1974) and in calf thymus (Weil and Blatti, 1975), although in these mammalian systems only one form of enzyme III was demonstrated. The divalent cation activity ratios for the HeLa enzymes were determined from cation titration curves (not shown). Mn^{2+}/Mg^{2+} activity ratios at optimal divalent cation concentrations for polymerases I, II, III_A, and III_B are 1.3, 5.9, 1.8, and 1.7, respectively. HeLa enzyme II is extremely sensitive to Mn^{2+} stimulation. These values are similar to those obtained in other mammalian systems (Schwartz et al., 1974; Austoker et al., 1974; Weil and Blatti, 1975).

α-Amanitin, the mushroom toxin derived from *Amantia phalloides*, differentially inhibits the three major classes of HeLa cell RNA polymerases. As Figure 4 shows, enzyme II is inhibited at very low concentrations of the mushroom toxin, with 50% inhibition of activity occurring at an α-amanitin concentration of 0.003 μg/ml. The class III enzymes, III_A and III_B, exhibit an intermediate sensitivity to α-amanitin with 50% inhibition of activity occurring at an amanitin concentration of about 15 μg/ml. Polymerase I is not inhibited significantly even at α-amanitin concentrations >400 μg/ml. The α-amanitin sensitivities of the four forms of HeLa RNA polymerases are relatively invariant (±5%) and do not change when different batches of α-amanitin from different manufacturers are used.

Functional Studies on the Class III HeLa Enzymes. Each of the major classes of HeLa enzymes retain their characteristic α-amanitin sensitivities even when assayed in vitro in nuclei. Three major regions of activity can be seen in Figure 5 which depicts the α-amanitin inhibition curve of total RNA synthesis in HeLa nuclei. Polymerase II activity is inhibited at low amanitin concentrations, polymerase III (III_A–III_B) at intermediate concentrations and polymerase I activity is resistant to inhibition at α-amanitin concentrations of >400 μg/ml. The amanitin concentration required for inhibition and relative amounts of the three classes of RNA synthetic activity varies from experiment to experiment (i.e., ±10–20%), but the shape of the α-amanitin titration curve does not change. It should be noted that the relative amounts of each of these enzymes shown here (I, 28%; II, 62%; III, 10%) do not accurately measure the amounts of each form present within the cell (see Tables I and IV). Except in the case of RNA polymerase which has been shown to reinitiate the synthesis of 5S RNA in vitro in nuclei (Marzluff et al., 1974), activity assayed in

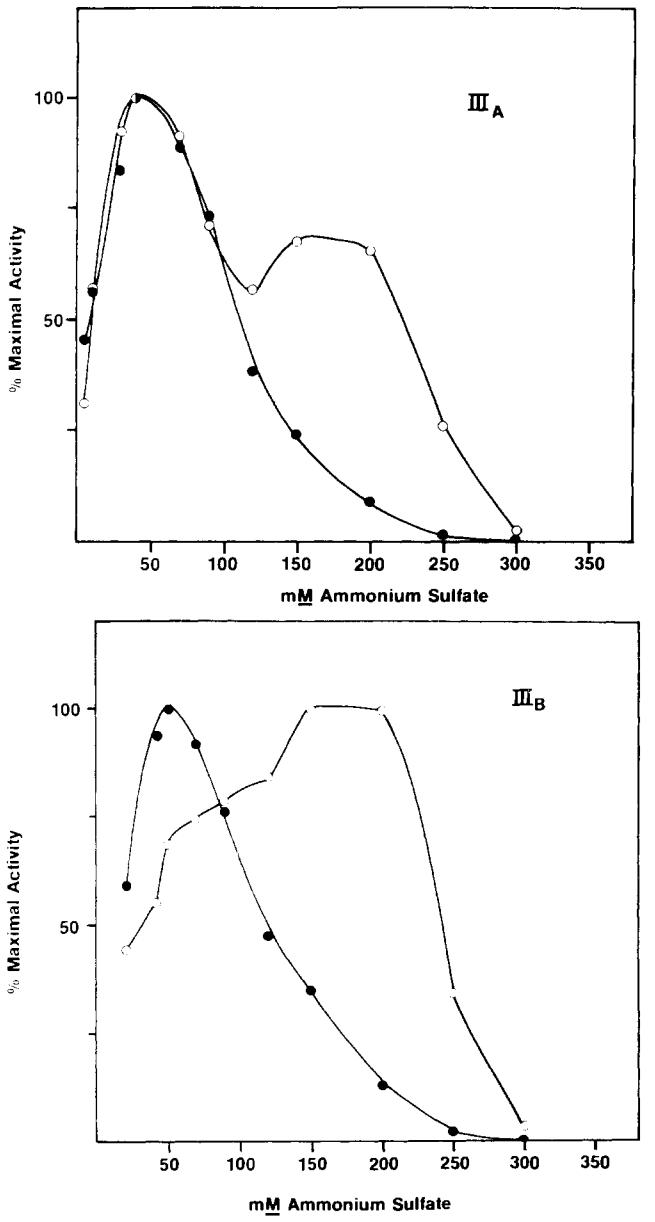

FIGURE 3: Ammonium sulfate titration curves of the class III enzyme forms of HeLa cells. Enzymes were purified as described in the text. Ten-microliter aliquots of each enzyme form were assayed at the indicated ammonium sulfate concentration. Enzymes were assayed with either calf thymus DNA (○) or poly[d(A-T)] (●) as template. Maximum activities for the individual enzymes with calf thymus DNA template were: III_A, 2800; and III_B, 2500 cpm. In the case of poly(d(A-T))-directed synthesis, maximum activities were: 5600 and 6200 cpm respectively for enzymes III_A and III_B.

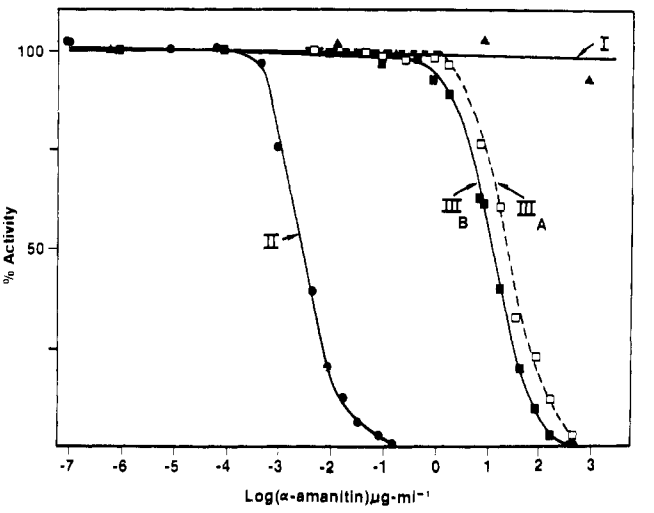

FIGURE 4: α-Amanitin sensitivities of the HeLa RNA polymerases. The source of enzymes was as described in the text. Enzymes were assayed at their respective ammonium sulfate optima with calf thymus DNA (see Figure 3), in the presence of increasing amounts of α-amanitin: (▲—▲) I; (●—●) II; (□- - -□) III_A; and (■—■) III_B α-amanitin inhibition curves. One hundred percent activity represents 18 300, 3200, 4600, and 6800 cpm respectively for enzymes I, II, III_A, and III_B.

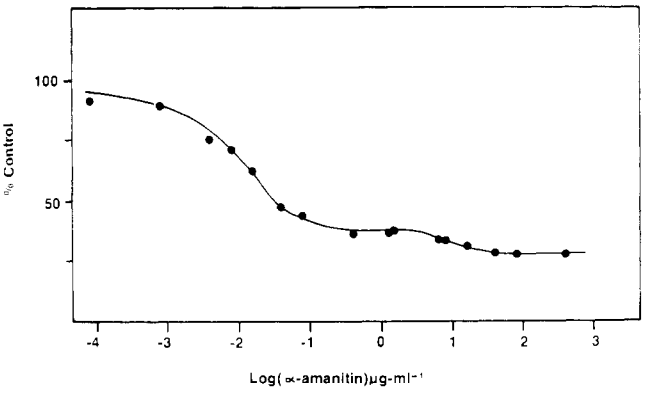

FIGURE 5: Effect of α-amanitin concentration on total RNA synthesis in HeLa nuclei in vitro. Nuclei from HeLa cells were prepared as described in the text and conditions of RNA synthesis were as described (Blatti et al., 1970). RNA was synthesized by nuclei in the presence of increasing concentrations of α-amanitin; [^{3}H]UMP incorporation into RNA after synthesis was determined by lysing nuclei with 0.5% sodium dodecyl sulfate and spotting the samples on DE-81 filters as described in the text. The amount of RNA synthesized in the control assay was 12 200 cpm. All points were done in triplicate. Each assay contained approximately 1×10^6 nuclei.

nuclei measures pre-initiated RNA polymerase. Therefore, exact quantitation of polymerases requires solubilization and DEAE-Sephadex chromatography.

The unique α-amanitin sensitivity of the class III enzymes and the observations by Penman and co-workers that the synthesis of the low-molecular-weight RNAs, 5.5S Adenovirus-2 specific RNA (Price and Penman, 1972) and pre-tRNA and 5S RNA (McReynolds and Penman, 1974a), were resistant to low levels of α-amanitin prompted us to study more closely the synthesis of pre-tRNA and 5S RNA in HeLa cells. As McReynolds and Penman (1974a) demonstrated, low-molecular-weight RNAs are released from HeLa nuclei when they are synthesized in vitro under favorable conditions. When these RNAs are analyzed on sucrose gradients, the in vitro RNAs sediment with, and slightly faster than, HeLa marker tRNAs (data not shown). Also no species with sedimentation coefficient >5S is found in sucrose gradients or 10% polyacrylamide gels. When subjected to electrophoresis on 10% acrylamide gels, these RNA species are found to be 5S RNA and the well characterized tRNA precursor, 4.5S RNA (Burdon, 1971; Bernhardt and Darnell, 1969; Bernhardt-Mowshowitz, 1970; Choe and Taylor, 1972; McReynolds and Penman, 1974a,b; Marzluff et al., 1974).

When low-molecular-weight RNAs are synthesized in vitro, in HeLa nuclei, in the presence of increasing concentrations of α-amanitin, one finds that the synthesis of 5S and 4.5S pre-tRNA is almost totally abolished by toxin concentrations around 130 μg/ml. The results of these experiments depicted in Figure 6 show the composite of four

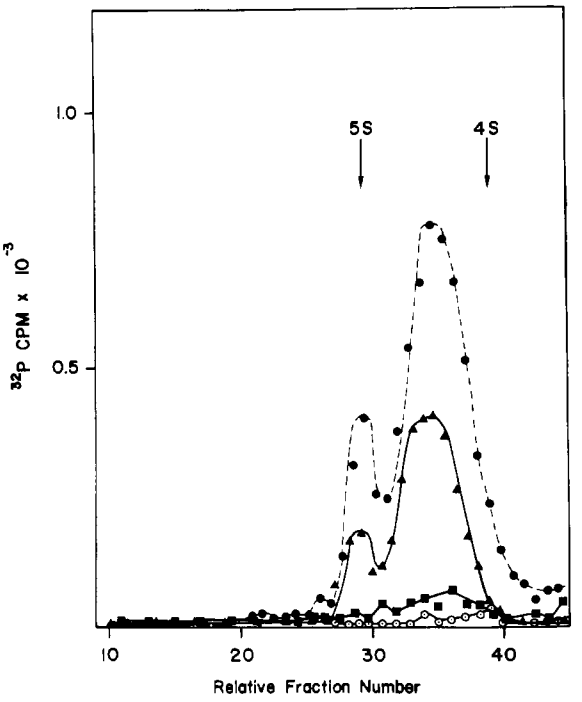

FIGURE 6: Effect of α-amanitin concentration on the amounts of pre-tRNA and 5S RNA synthesized in vitro by HeLa cell nuclei. Low molecular weight RNAs were synthesized in vitro in the presence of (●- -●) 0.64, (▲—▲) 6.4, (■—■) 64, or (⊙—⊙) 128 μg/ml of α-amanitin. ^{32}P-Labeled RNAs synthesized were separately purified by phenol extraction and ethanol precipitation as described in the text and analyzed on 10% polyacrylamide gels. The results of the separate experiments are plotted together for comparison, relative to 4S and 5S marker RNAs. The marker RNAs were included in each reaction mixture prior to the initiation of synthesis.

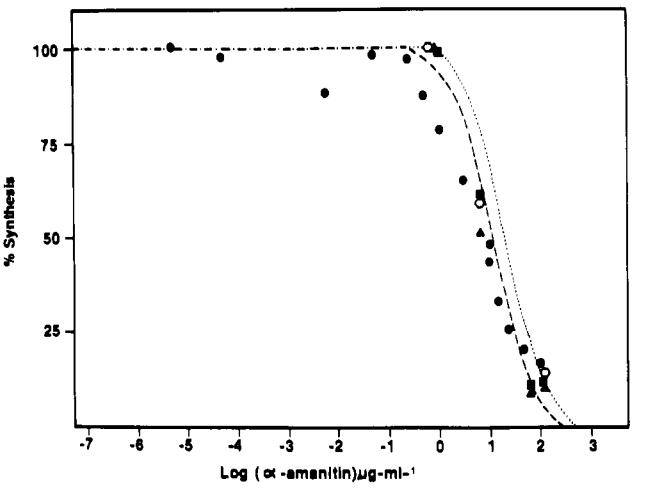

FIGURE 7: α-Amanitin titration curve for the inhibition of synthesis of pre-tRNA and 5S RNA in vitro. RNA was synthesized in the presence of the indicated amount of α-amanitin and analyzed by various procedures as described below, and the percent inhibition of synthesis was calculated. Data obtained from several experiments have been plotted together: (▲), 4.5S pre-tRNA and (■) 5S RNA as analyzed by 10% polyacrylamide gel electrophoresis (data obtained from Figure 6); (○), pre-tRNA *and* 5S RNA as analyzed on 5–20% sucrose gradients in buffer G; (●), pre-tRNA *and* 5S RNA monitored as trichloroacetic acid precipitable radioactivity present in the supernatant of reaction mixtures after RNA was synthesized in vitro in HeLa nuclei. The inhibition curves for the homologous HeLa class III enzymes are included for comparison: (. . .) enzyme III_A; (- - -) enzyme III_B. One hundred percent synthesis corresponds to: 18 000 cpm for the case of trichloroacetic acid precipitable pre-tRNA and 5S RNA; 12 000 cpm where pre-tRNA and 5S RNA were analyzed together by sucrose gradient centrifugation; and 3700 cpm in 4.5S pre-tRNA and 1050 cpm in 5S RNA where these species were analyzed separately on 10% polyacrylamide gels.

separate 10% polyacrylamide gel analyses plotted together relative to 4S marker RNAs. If the percent inhibition of in vitro synthesis of pre-tRNA and 5S RNA is plotted as a function of α-amanitin concentration, the graph in Figure 7 is obtained. The dashed and dotted lines represent the α-amanitin titration curves of the chromatographically purified HeLa polymerases III_A and III_B for comparison. The data from several experiments are included, where either 4.5S and 5S RNAs were analyzed (1) separately on 10% acrylamide gels, (2) together by sucrose gradient centrifugation, or (3) as radioactive RNAs found in the supernatant after synthesis of RNA in nuclei and monitored as trichloroacetic acid precipitable material. It is readily seen that the inhibition curves for the synthesis of pre-tRNA–5S RNA and the partially purified class III enzymes are essentially identical, with 50% inhibition of synthesis occurring at about 15 μg/ml of α-amanitin. In order to characterize the 4.5S RNA species as a bona fide transfer RNA precursor, we attempted to process it in vitro to a 4S RNA molecule. This processing of pre-tRNA has been described in a number of procaryotic (see review by Altman, 1975) and eucaryotic systems (Burdon, 1971; McReynolds, and Penman, 1974; Marzluff et al., 1974). Two major procedures have been used in the past by many investigators for the preparation of the crude processing enzymes and the conditions of in vitro cleavage. Bernhardt-Mowshowitz (1970) obtained the processing enzymes from the post-ribosomal supernatant of HeLa cells and the processing reactions were performed under high salt (0.27 M ammonium sulfate), high pH (9.0) conditions. Marzluff et al. (1974) obtained processing enzymes from a detergent wash of nuclei, and conditions of processing were pH 8 and 0.15 M NaCl. Processing of in vitro synthesized RNAs was performed using both methods. Using the procedure described by Bernhardt-Mowshowitz (1970), little if any processing occurred (data not shown). When processing was performed as described by Marzluff et al. (1974), complete processing of pre-tRNA occurred (Figure 8). The reasons for the lack of processing with the Bernhardt-Mowshowitz procedure are unknown, but if processing is carried out with the crude enzyme preparation prepared by the method of Marzluff under high salt conditions, little if any processing occurs (data not shown).

Discussion

In this paper we have described and characterized the three major classes of DNA dependent RNA polymerases present in HeLa cells. Most significantly, we find two forms of RNA polymerase III, designated III_A and III_B, in these cells. This result is in contrast to the data of Seifart and Benecke (1975) who report only one form of enzyme III (C) in HeLa cells. However, our findings are consistent with those of Roeder and co-workers who describe two forms of enzyme III in a number of mammalian systems (Schwartz et al., 1974). The question of whether a single form (Sergeant and Krsmanovic, 1973; Austoker et al., 1974; Weil and Blatti, 1975) or two forms of enzyme III exist in all mammalian cells awaits further experimentation.

We have characterized the HeLa enzymes III_A and III_B with respect to catalytic and chromatographic properties. The only truly distinguishing characteristic of the two enzymes is chromatographic behavior on DEAE-Sephadex

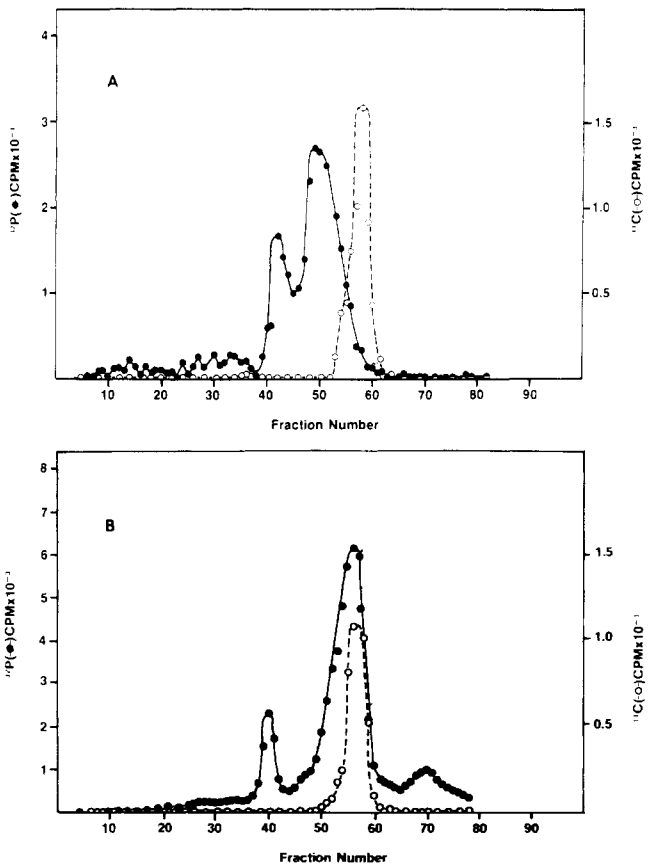

FIGURE 8: Processing of in vitro synthesized 4.5S pre-tRNA to 4S transfer RNA. Preparation of processing enzymes and conditions of maturation were as described (Marzluff et al., 1974). ^{32}P-Labeled substrate RNAs (containing both 4.5S pre-tRNA and 5S RNA) were synthesized in vitro and purified as described in the text. The final RNA precipitate was dissolved in 4 ml of 50 mM NaCl, 0.1 mM EDTA, and 0.5% sodium dodecyl sulfate and dialyzed overnite, with two changes, against 2 l. of the same solution. RNAs were precipitated with ethanol. The precipitate containing the substrate RNAs was recovered by low speed centrifugation, lyophilized, and dissolved in 400 μl of distilled water. In (A), 20 μl of ^{32}P-labeled substrate RNAs, mixed with 30 μl of ^{14}C-labeled marker tRNA and 50 μg of yeast carrier RNA, was incubated for 120 min at 37 °C with 750 μl of buffer F. In (B), 70 μl of the substrate RNAs was incubated with 500 μl of crude processing enzymes and the same amount of marker and carrier RNA for 90 min at 37 °C. Reactions were terminated by the addition of 1.5 ml of 50 mM NaCl, 0.1 mM EDTA, and sodium dodecyl sulfate to 1%. The samples were then dialyzed overnight against 4 l. of the same solution and subsequently phenol extracted and ethanol precipitated. The final RNA precipitates were lyophilized to dryness, redissolved in a minimum of 0.1-times concentrated electrophoresis buffer, and then analyzed on 10% polyacrylamide gels as described in the text. (O- - -O) Marker tRNA; (●—●) ^{32}P-labeled in vitro RNAs.

columns. Multiple forms of enzyme III (III_A and III_B) can be resolved in the HeLa system, but only when solubilized enzymes are chromatographed on DEAE-Sephadex. If cellular extracts are chromatographed on DEAE-cellulose, enzymes III_A and III_B elute at low ammonium sulfate concentrations, coincident with α-amanitin resistant RNA polymerase I. Enzymes III will thus not be detected unless the peak of amanitin resistant activity is titrated with α-amanitin or rechromatographed on DEAE-Sephadex (see Figures 1 and 2). The basis for this unusual chromatographic behavior is not known, but it has been observed in a number of mammalian systems (Sergeant and Krsmanovic, 1973; Austoker et al., 1974; Seifart and Benecke, 1975). Whether or not there are structural or charge differences between these two enzyme forms awaits purification to homogeneity and subsequent subunit structural analysis.

Enzymes III_A and III_B exhibit characteristic divalent cation optima and ammonium sulfate titration curves, properties which serve to distinguish the class III polymerases from the homologous enzymes I and II. When the HeLa class III enzymes are assayed for ammonium sulfate activation with calf thymus DNA, they exhibit broad biphasic titration curves similar to the *Xenopus laevis* (Roeder, 1974) and mouse myeloma (Schwartz et al., 1974) enzymes.

The most distinctive property of the HeLa class III enzymes is an intermediate sensitivity to α-amanitin inhibition, with 50% inhibition of activity of polymerases III_A and III_B occurring at an α-amanitin concentration of 15 μg/ml. This is greater than three orders of magnitude above the concentration required to inhibit HeLa polymerase II. As reported in other systems, enzyme I is resistant to α-amanitin inhibition even at very high toxin concentrations (>400 μg/ml). All three major classes of enzyme (I, II, and III) retain their characteristic α-amanitin sensitivities whether assayed in a solubilized state or, in vitro, in nuclei.

We have utilized these properties of the HeLa polymerases to titrate in isolated nuclei, in vitro the synthesis of pre-tRNA and 5S ribosomal RNA with α-amanitin. The α-amanitin inhibition curves for the synthesis of these low-molecular-weight RNAs and the α-amanitin inhibition curves for the chromatographically purified homologous enzymes, III_A and III_B, are essentially identical (50% inhibition at 15 μg/ml of α-amanitin). These data demonstrate that the class III enzymes are responsible for the transcription of pre-tRNA and 5S RNA in vivo. However, it is not presently clear whether (1) a particular form of enzyme III (III_A or III_B) is responsible for the transcription of a particular set of genes (pre-tRNA or 5S RNA genes) or (2) whether III_A and III_B are alternative forms of the same enzyme (e.g., initiation and elongation forms) which transcribe both sets of genes. These results confirm and extend our previous data and conclusions (Weil et al., 1974) and represent an independent confirmation of the in vivo function of the mammalian class III enzymes (see Weinmann and Roeder, 1974a,b; and Weinmann et al., 1974, 1975).

The precursor tRNA synthesized by nuclei can be processed, in vitro, to an RNA species co-migrating with bona fide tRNA *and* an additional small RNA fragment (estimated mol wt of 5000). On the other hand, the size of the 5S ribosomal RNA species was not detectably altered, as expected, upon treatment with processing enzymes (see Figure 8B).

Interestingly, in the HeLa system there does not appear to be exclusive localization of either enzyme III_A or III_B in the cytoplasm. This result is in contrast to the data of Schwartz et al. (1974) who report that enzyme III_B is found only in the cytoplasmic subfraction of mouse myeloma cells. Whether their result represents a case of selective nuclear leakage for enzyme III_B requires further study. However, transcription of the reiterated, clustered tRNA and 5S RNA genes present in the nucleus (Brown et al., 1971; Clarkson et al., 1973; Pardue et al., 1973) appears to preclude a major cytoplasmic function for the class III enzymes. Further studies correlating the relative proportions of pre-tRNA and 5S RNA synthesized by nuclei in vitro with the amounts of enzymes III_A and III_B actually present in those nuclei might help to determine whether or not the class III enzymes have discrete functional roles in mammalian cells. These studies are currently in progress in our lab-

oratory.

The class III enzymes have now been isolated and characterized in a number of eucaryotic systems, and purification to homogeneity is imminent (Sklar et al., 1975). Therefore, a new model transcription system utilizing purified enzymes (RNA polymerase III) and defined templates (e.g., bacterial plasmids carrying the purified genes for tRNA or 5S RNA) for elucidating the complex problem of the control of eucaryotic gene expression by enzyme molecules, putative eucaryotic transcription factors, and chromosomal proteins is now potentially available.

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