On the DNA Polymerase III of Mouse Myeloma: Partial Purification and Characterization†

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ABSTRACT: A high molecular weight membrane-bound DNA polymerase from the mouse myeloma, MOPC-104E, has been purified extensively, and characterized with regard to physical and reaction properties. This enzyme, which is readily distinguishable from other myeloma enzymes that are analogous to the recognized forms of cellular DNA polymerase, is designated DNA polymerase III. DNA polymerase III activity in whole homogenates from MOPC-104E was solubilized and then purified using a series of ion-exchange chromatographic procedures followed by DNA-cellulose chromatography and glycerol gradient centrifugation; the enzyme activity, as measured with poly(rA) · $(dT)_{12-18}$ as template-primer and Mn^{2+} as divalent cation, was purified as much as 18,000-fold. In the final stages of the purification, DNA polymerase III possessed no detectable RNA polymerase activity, nucleoside diphosphokinase activity, or nuclease activity toward DNA or single- and double-stranded RNA. Ribonuclease H activity was present through most of the purification. However, this activity did not precisely copurify with the DNA polymerase activity, and was completely separated from DNA polymerase III in the final step of the purification. The isoelectric point of the purified DNA polymerase III was approximately pH 5.8; the approximate molecular weight under nondissociating conditions was 315,000 by gel filtration, 270,000 by gel electrophoresis, and 230,000 by sedimentation velocity (relative to catalase at 243,000). As revealed by glycerol gradient centrifugation, the enzyme was not dissociated into smaller species possessing activity by treatment with either 125 mM ammonium sulfate, RNase and DNase, or 500 mM KCl and 0.2% Tween-80. In solutions of relatively low ionic strength and under certain other conditions, the enzyme aggregated into a much higher molecular weight species. Activity of DNA polymerase III was inhibited by sulfhydryl-blocking reagents and by 1,10-phenanthroline; it was not inhibited by antiserum directed against MuLV DNA polymerase. DNA polymerase III required a base pair complementary combination of template, primer, and deoxynucleoside 5′-triphosphate for activity; as template it preferred poly(rA), poly(dA), and poly(dC), and was relatively inactive with calf thymus DNA, poly(rI), poly(rC), and poly(dG) under the conditions tested. The template specificity varied depending upon whether Mn^{2+} or Mg^{2+} was the divalent cation. Several properties of the polymerase activity such as pH optima, activation energy, etc., were different in reactions with poly(rA) · $(dT)_{12-18}$ and Mn^{2+} as divalent cation than in reactions with poly(dA) · $(dT)_{12-18}$ and Mg^{2+} as divalent cation. This suggests that the precise nature of the enzyme reaction under the two conditions is not identical, yet the active sites appear to reside in the same protein complex. Adult BALB/c mouse liver was found to contain a DNA polymerase with chromatographic and sedimentation properties virtually identical with those of the MOPC-104E DNA polymerase III. This liver enzyme was not detected in either purified mitochondria or in the cytoplasmic S100 fraction. Thus, an enzyme that appears analogous to the myeloma DNA polymerase III is present in a normal mouse tissue.

As a first step in our study of mechanisms of mammalian DNA synthesis, the DNA polymerase content of the rapidly growing yet highly specialized (IgM producing) mouse myeloma, MOPC-104E, was examined. We found that DNA polymerase activity solubilized from whole cell homogenates could be reproducibly separated into five distinct molecular forms that exhibited relatively abundant activity (Matsukage *et al.*, 1974a). The precise physicochemical relationship among these forms of DNA polymerase is a matter of current investigation.

Two of the forms of MOPC-104E DNA polymerase appeared to be analogous to the high and low molecular weight forms of DNA polymerase previously recognized in many other eucaryotic cell types[1] (Chang and Bollum, 1972a; Loeb, 1974). A third enzyme, which possessed abundant activity among the myeloma DNA polymerases, has been designated DNA polymerase III (DPase III);[2] this en-

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[1] In addition to mitochondrial-associated enzymes, we consider two forms of DNA polymerase as well recognized in mammalian cells: DNA polymerase I designates an enzyme with an isoelectric point of pH 5.4 that is present in the cytoplasmic supernatant fraction and sediments at approximately 6 S in glycerol gradients containing 500 mM KCl. It is active with activated calf thymus DNA and poly[d(A-T)] as template-primer. Under the conditions used it is tightly bound to DEAE-cellulose columns at pH 7.7, and is less tightly bound to phosphocellulose columns at pH 6.8 than the other myeloma DNA polymerases. DNA polymerase II designates an enzyme with an isoelectric point of pH 9.4 that sediments at approximately 2.5 S–3.5 S in glycerol gradients containing 500 mM KCl. It is active with both poly(rA) · $(dT)_{12-18}$ and activated calf thymus DNA as template-primer. Under the conditions used, it is not bound to DEAE-cellulose columns at pH 7.7, but is more tightly bound to phosphocellulose columns at pH 6.8 than any of the other myeloma DNA polymerases.

[2] Abbreviations used are: DNA polymerase and DPase, deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7; RNase H, ribonuclease H; DNase, deoxyribonuclease; MuLV, murine leukemia virus; KP_i, potassium phosphate buffer; DEAE-cellulose, diethylaminoethylcellulose; PC, phosphocellulose; HA, hydroxylapatite, DTT, dithiothreitol; BPA, bovine plasma albumin; SDS, sodium dodecyl sulfate; MOPC-104E, mineral oil plasmacytoma; S.S. DNA, salmon sperm DNA; C.T. DNA, calf thymus DNA; SV40 DNA, simian virus 40 DNA. Abbreviations for nucleotides and synthetic polynucleotides are according to the IUPAC-IUB Commission (1970).

zyme was localized primarily in the cytoplasmic membrane fraction, sedimented in glycerol gradients faster than bovine serum albumin in the range of approximately 6 S–8 S, and was much more active with poly(rA) · $(dT)_{12-18}$ as template–primer than with activated calf thymus DNA.

The purpose of this report is to describe the partial purification and characterization of the MOPC-104E DPase III, and the detection of an enzyme with virtually identical chromatographic and sedimentation properties in normal mouse liver.[3] As in the case of myeloma DPase III, the analogous enzyme in liver possessed abundant activity among the liver DNA polymerases, was localized in the cytoplasmic membrane fraction, and was distinguishable from both the high molecular weight cytoplasmic supernatant fraction and low molecular weight forms of DNA polymerase present in liver. The results are in agreement with suggestions that DPase III is a distinct new cellular DNA polymerase and they provide more information for comparison of the enzyme with various other mammalian DNA polymerases.

It is important to note that it was originally unclear to us whether the poly(rA)-directed DNA polymerases described by Chapeville and coworkers (Maia *et al.*, 1971), Weissbach and coworkers (Fridlender *et al.*, 1972; Bolden *et al.*, 1972), and others were novel polymerases or were forms of recognized enzymes such as the low molecular weight cellular DNA polymerase II. This confusion occurred because the molecular weight and certain other physical properties of the poly(rA)-directed DNA polymerases were not reported, and also because the low molecular weight DNA polymerase isolated by Weissbach and coworkers was not characteristic of the previously defined DNA polymerase II (Chang and Bollum, 1972b) in that it was not active with poly(rA) as template and was not found in the cytoplasmic fraction as well as in the nuclear fraction. The possible relationship between the DPase III of the current work and other poly(rA) reading DNA polymerase is discussed.

Experimental Section

Materials

The myeloma, MOPC-104E, in solid tumor was originally obtained from Dr. M. Potter, National Institutes of Health. It was passaged subcutaneously in female BALB/c mice as described by Wilson *et al.* (1974). Livers were obtained from female BALB/c mice 12–14 weeks of age. Oligonucleotides were from Collaborative Research, Inc. Synthetic polynucleotides were from Miles Laboratories; unlabeled nucleotides were from Calbiochem; radioactive nucleotides were from Schwarz/Mann. Native calf thymus (C.T.) DNA and salmon sperm (S.S.) DNA were from Worthington Biochemical Corp. and Calbiochem, respectively; they were activated as template–primers for the activity of *Escherichia coli* DNA polymerase I by digestion with beef pancreas DNase I according to the method described by Aposhian and Kornberg (1962). Moloney MuLV 70S RNA (Tsuchida *et al.*, 1972) was kindly provided by Dr. N. Tsuchida, Flow Laboratories. Mouse liver messenger RNA was prepared by the method described by Aviv and Leder (1972). SV40 DNA and *E. coli* RNA polymerase were kindly provided by Dr. P. Qasba, National Institutes of Health. *Aspergillus oryzae* nuclease S1 (Ando, 1966) was prepared according to the method described by Sutton (1971). Radioactive RNA · DNA hybrids for use as substrates in RNase H assays were prepared using *E. coli* RNA polymerase according to the method described by Grandgenett *et al.* (1973). ^{3}H-labeled T7 phage DNA was prepared as described by Matsukage (1972). ^{3}H-labeled *E. coli* total RNA was prepared as described by Matsukage and Minagawa (1969). Bovine pancreatic trypsin, RNase B and DNase I, bovine liver catalase, bovine heart lactate dehydrogenase, and hen egg-white lysozyme were from Worthington Biochemical Corp. ^{125}I (0.73×10^6 cpm/pmol) was from Amersham-Searle. Bovine plasma albumin was from Armour; ^{125}I-labeled bovine plasma albumin (0.64×10^5 cpm/μg) was prepared by the method described by Onoue *et al.* (1964). B-Phycoerythrin was kindly provided by Dr. R. Gantt, National Institutes of Health. DNA-cellulose was prepared according to the method described by Litman (1968), using native calf thymus DNA and 100–250 mesh wood cellulose, seived from Solka-Floc SM-40-B, obtained from the Brown Co., Berlin, N.H. After the DNA-cellulose had been prepared and washed as described (Litman, 1968), it was incubated at 24° for 15 min with 0.01 μg/ml of bovine pancreatic DNase I in 50 mM Tris-HCl (pH 7.7)[4] containing 1 mM $MgCl_2$. The suspension was then adjusted to 7 mM in EDTA, the DNA-cellulose was collected on a glass-fiber filter and washed first with 50 mM Tris-HCl (pH 7.7), 1 mM EDTA, and 600 mM KCl, and next with 50 mM Tris-HCl (pH 7.7) and 1 mM EDTA. The DNA-cellulose was then poured into 0.6×5 cm columns and washed with 50 mM Tris-HCl (pH 7.7), 1 mM DTT, 1 mM EDTA, and 20% glycerol. *E. coli* B DNA polymerase I, fraction VII, was from General Biochemicals; Rauscher murine leukemia virus (MuLV) was from Electro-Nucleonics, Inc. Rabbit antiserum directed against Rauscher MuLV DNA polymerase (Aaronson *et al.*, 1971) was kindly provided by Drs. E. Scolnick and W. Parks, National Institutes of Health. *o*- and *m*-Phenanthroline were from Fisher Scientific and ICN-K & K Laboratories, Inc., respectively. Diethylaminoethylcellulose (DE-52, microgranular) was from Whatman Biochemicals, Ltd; phosphocellulose (0.9 mequiv/g) was from Sigma Chemical Co., and hydroxylapatite (Hypatite C; maximum capacity of 78 mg of albumin/g) was from Clarkson Chemical Co., Inc.

Methods

Assays for DNA Polymerase Activity. DNA polymerase activity was measured by determining the rate of conversion of ^{3}H-labeled deoxynucleoside 5′-triphosphates into cold 10% trichloroacetic acid insoluble material as a result of incubation with enzyme. Unless indicated otherwise, reactions were incubated at 37° for 60 min in a final volume of 25 μl in 10×75 mM siliconized soft glass tubes and contained the following:[3] 55 mM Tris-HCl (pH 7.8–7.9) at 37°, 1.3 μM [*methyl*-^{3}H]dTTP (17.5 Ci/mmol), either 0.5 mM $MnCl_2$ or 4 mM magnesium acetate as indicated, 80 mM KCl, 15% (v/v) glycerol, 400 μg/ml of bovine plasma albumin, 160 μg/ml of either poly(rA) or poly(dA) as indicated, 32 μg/ml of $(dT)_{12-18}$, 0.1 mM EDTA, 1.1 mM DTT, and the indicated amount of enzyme protein.

Reactions were terminated by transferring the tubes to Dry Ice and then to a 0–1° ice–water bath. To each reaction mixture 100 μg of bovine plasma albumin was added,

[3] Preliminary reports of these findings have been published (Matsukage *et al.*, 1974b,c).

[4] The concentrations and pH values refer to the final solution and temperature used.

followed by 1.5 ml of solution T [100% Cl_3CCOOH, 10 mM thymidine, saturated sodium pyrophosphate, saturated sodium orthophosphate (1:1:4:4)] at 4°. Contents of each tube were mixed and after 15 min at 0–1°, insoluble material was collected on a nitrocellulose filter (Millipore, 0.45 μ pore size) and washed on the filter with approximately 40 ml of 10% Cl_3CCOOH at 4° followed by 20 ml of chloroform–methanol (1:1) at 4°. Each filter was then transferred to a glass scintillation vial and dissolved in 1 ml of methyl Cellosolve at 24°; 10 ml of Triton X-100–toluene–Permafluor (Packard) (32:64:4) scintillation mixture was added, and after thorough mixing radioactivity was determined in a scintillation spectrometer (Schrier and Wilson, 1973) at a counting efficiency of 30%. Results of enzyme assays, expressed as (Δ) pmol of ^{3}H-labeled deoxynucleoside 5′-monophosphate incorporated per reaction, indicate the enzyme-dependent change in cold acid-insoluble radioactivity. The Δ pmol value represents the difference between the amount of incorporation in the presence of enzyme and the amount of incorporation in the absence of enzyme. In typical reactions containing no enzyme incorporations were 0.003–0.006 pmol of ^{3}H-labeled deoxynucleotide 5′-monophosphate. One unit of DNA polymerase activity is equal to 1 Δ pmol of [^{3}H]dTMP incorporated per 60 min at 37°.

Assay for Ribonuclease H Activity: The assay described is a modification of the method of Grandgenett *et al.* (1973); conversion of radioactive RNA in a RNA–DNA hybrid into cold acid-soluble material was measured. Reactions were incubated at 37° for 5–60 min in a final volume of 25 μl and contained the following: 50 mM Tris-HCl (pH 7.7), 19% glycerol, 400 μg/ml of bovine plasma albumin, 2 mM $MnCl_2$, 80 mM $(NH_4)_2SO_4$, 16 mM NaCl, 72 mM KCl, 0.2 mM EDTA, 1.2 mM DTT, either 0.1 pmol of [^{3}H]AMP in the form of ^{3}H-labeled $(rA)_n \cdot (dT)_n$ (18,000 cpm/pmol) or 6 ng of ^{3}H-labeled $(dT)_n$ (5000 dpm/ng) and 60 ng of poly(rA), and 5–20 ng of enzyme protein. After incubation, 100 μg of bovine plasma albumin was added to each tube; reactions were adjusted to 10% in CCl_3COOH in a final volume of 100 μl, allowed to stand for 15 min at 0–1°, and then centrifuged at 15,000*g* for 20 min; 75 μl of the supernatant fluid was withdrawn from each tube, placed in a glass scintillation vial along with 10 ml of Triton X-100–toluene–Permafluor scintillation mixture, and counted in a scintillation spectrometer at a counting efficiency of 45%.

Other Enzyme Assays. Assays for DNA endonuclease activity, performed in collaboration with Dr. P. Qasba, measured the conversion of SV40 DNA component I into component II (Sebring *et al.*, 1971; Qasba, 1974) during incubation with 30 ng of enzyme protein at 37° for 60 min.

Assays for DNase activity measured the conversion of native ^{3}H-labeled T7 DNA into cold acid-soluble material. Reactions were incubated at 37° for 60 min in a final volume of 100 μl and contained the following: 55 mM Tris-HCl (pH 7.7), 30 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 0.43 μg of ^{3}H-labeled T7 phage DNA (153,000 dpm/μg), and 30 ng of enzyme protein. After incubation, 100 μg of bovine plasma albumin and then 1.5 ml of 10% CCl_3COOH at 0–1° were added to each reaction. Reaction mixtures were allowed to stand for 15 min at 0–1°; acid-insoluble material was collected and washed on a nitrocellulose filter, and radioactivity was determined as described for the DNA polymerase assays.

Assays for RNA polymerase activity were performed under five different reaction conditions and measured the incorporation of [^{3}H]ATP into cold acid-insoluble material. Reaction mixtures under all conditions were incubated at 37° for 60 min and contained 15 ng of enzyme protein, 0.2 mM each CTP, GTP, and UTP, and 0.22 mM [^{3}H]ATP (8400 dpm/pmol); the other components for various reaction conditions (A–E) were as follows: (A) (Burgess, 1969) 40 mM Tris-HCl (pH 7.9), 2% glycerol, 0.2 mM DTT, 0.1 mM EDTA, 10 mM $MgCl_2$, 150 mM KCl, 500 μg/ml of bovine plasma albumin, and 150 μg/ml of calf thymus DNA in a final volume of 50 μl; (B) (Downey *et al.*, 1973) 80 mM Tris-HCl (pH 7.8), 2% glycerol, 1.1 mM DTT, 1 mM EDTA, 1.6 mM $MnCl_2$, 200 mM KCl, 400 μg/ml of bovine plasma albumin, and 20 μg/ml of mouse liver messenger RNA in a final volume of 50 μl; reactions under C conditions were identical with B conditions except that the KCl concentration was 30 mM; (D) (Meilhac *et al.*, 1972) 80 mM Tris-HCl (pH 7.9), 12% glycerol, 0.15 mM DTT, 0.1 mM EDTA, 3 mM $MnCl_2$, 50 mM ammonium sulfate, 15 mM KCl, 200 μg/ml of bovine plasma albumin, and 200 μg/ml of calf thymus DNA in a final volume of 100 μl; and (E) (Stein and Hausen, 1970) 30 mM Tris-HCl (pH 7.8), 1.3% glycerol, 0.07 mM DTT, 0.07 mM EDTA, 1 mM $MnCl_2$, 20 mM KCl, 400 μg/ml of bovine plasma albumin, and 100 μg/ml of calf thymus DNA in a final volume of 75 μl. Cold acid-insoluble radioactivity was collected and washed on nitrocellulose filters, and measured as described for the DNA polymerase assays.

Assays for ribonuclease activity were performed under three different reaction conditions and measured the conversion of total *E. coli* [^{3}H]RNA into cold acid-soluble material. All reactions were incubated for 60 min at 37° in a final volume of 50 μl and contained 13% glycerol, 0.06 mM DTT, 0.06 mM EDTA, 18 mM KCl, 0.6 mM magnesium acetate, 84.6 μg/ml of [^{3}H]RNA (52,000 dpm/μg), and 9 ng of enzyme protein. The other components for the various reaction conditions (A–C) were as follows: (A) 50 mM Tris-HCl (pH 8.0); (B) 50 mM Tris-HCl (pH 9.5); and (C) 50 mM sodium acetate (pH 5.2). After incubation, 100 μg of bovine plasma albumin and then 1.5 ml of 10% CCl_3COOH at 0–1° were added to each reaction. Reactions were then allowed to stand at 0–1° for 15 min before the cold acid-insoluble radioactivity was collected on nitrocellulose filters and measured.

The assay for nucleoside diphosphokinase activity measured the conversion of [^{3}H]dTDP into [^{3}H]dTTP using DNA polymerase that was dependent upon dTTP as a substrate. Reactions were incubated at 37° for 60 min in a final volume of 25 μl and contained 50 mM Tris-HCl (pH 8), 15% glycerol, 400 μg/ml of bovine plasma albumin, 32 μg/ml of $(dT)_{12-18}$, 160 μg/ml of poly(rA), 0.5 mM $MnCl_2$, 80 mM KCl, 1.1 mM DTT, 0.1 mM EDTA, 0.1 mM ATP, 1 μM [^{3}H]dTDP (8,360 dpm/pmol), and 1.5 ng of myeloma DNA polymerase III protein. Incorporation of radioactivity into DNA was measured as described above for the DNA polymerase assay.

Polyacrylamide Gel Electrophoresis. Electrophoresis under nondissociating conditions was performed by the method of Brown (1969) except that 4, 5, and 6% polyacrylamide gels were used. After electrophoresis at 10 mA/gel for 6 hr at 4°, gels (0.5 × 6 cm) were cut into slices 0.2 cm thick. Each slice was mixed with 75 μl of 50 mM Tris-HCl (pH 7.7), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 20% glycerol, and 1 mg/ml of bovine plasma albumin and allowed to stand for 16 hr at 0–1°; 10-μl portions of the solution with each slice were used for assay of DNA polymerase. The molecular weight of DPase III activity was deter-

Table I: Purification of Myeloma MOPC-104E DNA Polymerase III.

Step and Abbreviation	Total Protein Recovered (mg)	DNA Polymerase			
		Total Activity[b] Recovered (%)		Specific Activity[c]	Fold Purification
Crude extract	3525	208	100	0.059	1.0
30K*g* supernatant	2270	204	97.8	0.090	1.5
25–65% $(NH_4)_2SO_4$ fraction	1590	145	69.7	0.091	1.6
DEAE-cellulose (D3)	498	87	42.0	0.176	3.0
Phosphocellulose (D3P3)	15.2	193	93	12.7	215
Hydroxylapatite (D3P3H1)	3.3	134	64.5	40.6	689
DNA-cellulose (D3P3H1DC)	0.03[a]	31	15	1030	18,500
Glycerol gradient centrifugation	0.003[a]	1.2	0.4	406	6,890

[a] The amount of protein was measured by ^{125}I iodination as described under Methods. [b] Δnmoles of [^{3}H]dTMP incorporated per 60 min in reactions performed with poly[(rA)·$(dT)_{12-18}$]Mn^{2+} as described under methods, except that the dTTP concentration was 0.5 mM. [c] Δnmoles of [^{3}H]dTMP incorporated per 60 min per mg of protein.

mined by comparing the change in its mobility with different gel concentrations with the change in mobility of the marker proteins, catalase (243,000) and plasma albumin (68,000) (Hedrick and Smith, 1968).

Determination of Isoelectric Point. Isoelectrofocusing was performed according to the method described by Godson (1970) using 8.1-ml 1% ampholine gradients (pH 3–10) that were formed in 3–45% linear sucrose gradients containing 5 mM 2-mercaptoethanol and 5% glycerol. Electrofocusing was at 4° for 5 hr at an initial voltage of 500 V.

Measurement of Protein Concentrations: Protein was routinely measured by the method of Lowry *et al.* (1951) using bovine plasma albumin as standard. In some cases, however, this method was not effective due to very low concentrations of protein in enzyme preparations. For measurement of less than 1 μg of protein per assay, the capacity of unknown samples to affix ^{125}I was determined; the results were expressed as amount of protein relative to the standard, bovine plasma albumin. Iodination was performed using chloramine T and carrier free ^{125}I by the method of Onoue *et al.* (1964).

Preparation of Mouse Liver DNA Polymerase I and II. The high molecular weight liver DNA polymerase I (DPase I) was prepared according to the method described by Chang and Bollum (1972a). The supernatant fraction equivalent to the 100,000*g* (60 min) supernatant (Chang and Bollum, 1972a) was prepared by centrifugation of the cytoplasmic fraction at 78,000*g* for 90 min; this fraction is termed cytoplasmic 100K*g* supernatant. Protein in the fraction was precipitated in the presence of ammonium sulfate at 65% saturation, and the precipitate was collected by centrifugation at 15,000*g* for 20 min and dissolved in 0.1 M KP_i (pH 6.8) and 1 mM DTT.

DNA polymerase II (DPase II) was extracted from whole mouse liver as described for the preparation of the extract containing DNA polymerase III, except that DPase II did not bind to the DEAE-cellulose column and was recovered in the flow-through fraction during the wash with solution A containing 25 mM KCl. The DEAE-cellulose column fractions containing DPase II activity were pooled; protein was precipitated in the presence of ammonium sulfate at 65% saturation, recovered by centrifugation, and dissolved in solution B containing 50 mM KP_i (pH 6.8).

Preparation of Subcellular Fractions from Mouse Liver. Freshly excised BALB/c mouse livers were minced, mixed with two volumes of 0.25 M sucrose–1 mM EDTA, and homogenized using five strokes of a loose-fitting Teflon–glass homogenizer. The homogenate was filtered through cheesecloth and then separated into nuclear and cytoplasmic fractions by centrifugation at 600*g* for 12 min. Mitochondria were isolated and purified from the crude cytoplasmic fraction according to the method described by Mukerji and Deutscher (1972). The final mitochondrial preparation was free of contamination by membranes, debris, and nuclei, as judged by phase contrast light microscopy. The mitochondrial preparation was stored in pellet form in liquid nitrogen until use.

For preparation of the postmitochondrial membrane fraction, the supernatant obtained from the first centrifugation of the cytoplasmic fraction during the isolation of mitochondria was used. This supernatant fraction was centrifuged a second time at 8500*g* for 12 min and the resulting supernatant fluid was then centrifuged at 78,000*g* for 3 hr in a Spinco 30 rotor. The resulting pellet was stored in liquid nitrogen until use.

Results

Purification and Physical Properties of DPase III. The purification of DPase III was monitored by assay of DNA polymerase activity using poly(rA) · $(dT)_{12-18}$ as the template–primer and Mn^{2+} as the divalent cation. Data showing the typical recovery and purification of DNA polymerase III activity are summarized in Table I.

The enzyme-containing extract, prepared from an unfractionated homogenate of MOPC-104E, was applied to a DEAE-cellulose column and the column was then developed by stepwise additions of solutions containing increasing concentrations of KCl (Matsukage *et al.*, 1974a). The DPase III activity eluted in a single peak after the KCl concentration in the eluting solution was increased from 100 to 250 mM. Column fractions containing the enzyme were designated D3 as in the previous study (Matsukage *et al.*, 1974a). These fractions were pooled; protein in them was first concentrated by ammonium sulfate precipitation and then, following dialysis, was subjected to phosphocellulose column chromatography using a linear gradient of KP_i for elution (Figure 1A). Three prominent peaks of DNA polymerase activity were observed in the phosphocellulose col-

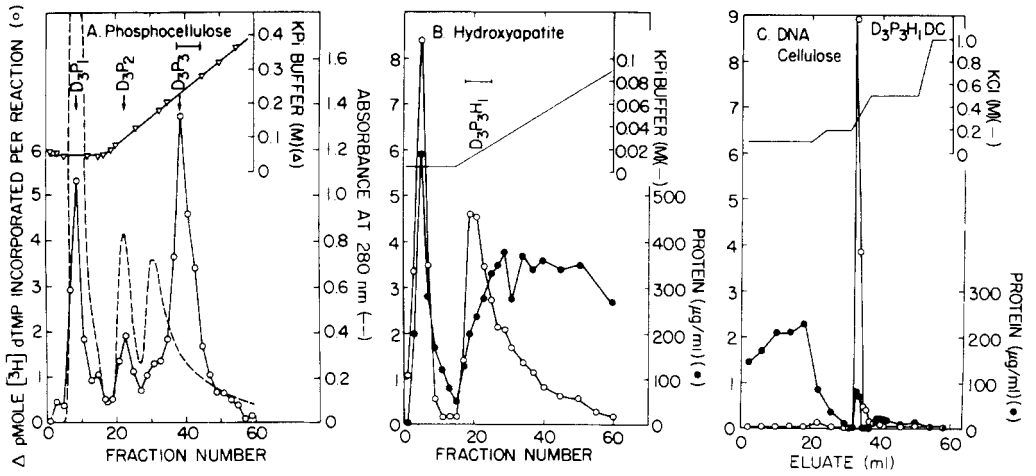

FIGURE 1: Purification of DNA polymerase III by column chromatography on phosphocellulose (A), hydroxylapatite (B), and DNA-cellulose (C); in each panel DNA polymerase activity is shown by the open circles and protein by either solid circles or broken line. Panel A shows the phosphocellulose column chromatogram of the 250 mM KCl-eluate from the DEAE-cellulose column. After addition of 250 mM KCl, the DEAE-cellulose column fractions containing poly(rA) · $(dT)_{12-18}$-dependent DNA polymerase activity were pooled (fraction "D3"), proteins were precipitated by ammonium sulfate at 65% saturation, and then dissolved in 4 ml of PC solution (50 mM KP_i (pH 6.8) containing 1 mM dithiothreitol and 20% glycerol). After dialysis against PC solution for 16 hr at 4°, a 3-ml portion containing 62.4 mg of protein was applied to a 1 × 15 cm phosphocellulose column previously washed and equilibrated with PC solution. The column was then washed with 35 ml of PC solution and developed with a 140-ml 50–700 mM linear gradient of KP_i containing 1 mM dithiothreitol and 20% glycerol (pH 6.8). Flow rate was 6 ml/hr and each fraction contained 2 ml. The DNA polymerase activity in 5-μl portions of fractions was assayed using poly(rA) · $(dT)_{12-18}$ and Mn^{2+} as described under Methods, except that [^{3}H]dTTP concentration was 0.5 mM (220 cpm/pmol). The fractions containing activity that eluted at approximately 240 mM potassium phosphate buffer were pooled, designated as "D3P3," and stored in 50 mM Tris-HCl (pH 7.7), 1 mM dithiothreitol, and 50% glycerol at −20°. Panel B shows the hydroxylapatite column chromatogram of DNA polymerase activity in fraction D3P3. The fraction D3P3 was dialyzed against HA solution (1 mM EDTA, 1 mM dithiothreitol, 500 mM KCl, 20% glycerol, and 0.2% Nonidet P-40) containing 5 mM KP_i (pH 7.0) for 16 hr at 4° and a portion containing 30 mg was then applied to a 1.0 × 15 cm hydroxylapatite column previously equilibrated with HA solution containing 5 mM KP_i (pH 7.0). The column was washed with 50 ml of the HA solution containing 5 mM KP_i, and developed with a 200-ml 5 mM (pH 7.0) to 100 mM (pH 6.4) linear gradient of KP_i in HA solution. Flow rate was 14 ml/hr, and each fraction contained 3.5 ml. The DNA polymerase activity in 1-μl portions of fractions was assayed as described under Methods; protein concentrations were determined by the method of Lowry *et al.* (1951). The recovery of enzyme activity was 86%. Fractions indicated were pooled and designated as D3P3H1. Panel C shows the DNA-cellulose column chromatogram of DNA polymerase fraction D3P3H1. The fraction designated D3P3H1 was dialyzed against DC solution (50 mM Tris-HCl (pH 7.7) containing 1 mM DTT, 1 mM EDTA, and 20% glycerol) for 16 hr at 4° and a portion containing 5.1 mg of protein was applied to a 0.6 × 14 cm DNA-cellulose column which had been previously equilibrated with DC solution. The column was washed with DC solution and then developed with stepwise additions of DC solution containing 200, 500, and 1000 mM KCl. The DNA polymerase activity in 1-μl portions of each fraction was assayed as described under Methods except that the incubation time was 30 min; protein concentrations were determined by the method of Lowry *et al.* (1951); 23% of the DNA polymerase activity present in fraction D3P3H1 was recovered during this step in the purification; of the 77% inactivation, 62% occurred during dialysis and 15% during the DNA-cellulose chromatography. The fraction corresponding to the peak of activity was designated D3P3H1DC.

umn chromatogram. These peaks were designated on the basis of their elution position as D3P1, D3P2, and D3P3.

The peak of poly(rA) · $(dT)_{12-18}$-dependent activity designated D3P2, which was eluted at 114 mM KP_i, was also found to be active with activated calf thymus DNA as template-primer; this activity was found to sediment at 6.1 S in glycerol gradients and was localized in the cytoplasmic high-speed supernatant fraction (Matsukage *et al.*, 1974a). The enzyme therefore appeared to correspond to the high molecular weight DNA polymerase I observed in a number of other mammalian tissues (Loeb, 1974).

The third peak of activity, designated D3P3, was eluted from the column at 240 mM KP_i and contained the DPase III activity. Fractions corresponding to the D3P3 peak of activity were pooled and protein in them was precipitated by adjusting the solution to 65% saturation in ammonium sulfate. The precipitate was collected by centrifugation and dissolved in a small volume of 50 mM Tris-HCl (pH 7.7), 500 mM KCl, 1 mM DTT, and 50% (v/v) glycerol. Enzyme activity in this solution was stable for 1 month at −20°. Hydroxylapatite column chromatography in the presence of 0.2% Nonidet P40 and 500 mM KCl was used in the next step of the purification (Figure 1B). A peak of DNA polymerase activity was observed in the chromatogram (Figure 1B) just after the start of the KP_i elution gradient. Fractions corresponding to this peak were pooled as shown in Figure 1B, designated D3P3H1, and stored in liquid nitrogen where enzyme activity was stable for at least 6 months.

Affinity Chromatography. The next step in the purification of DNA polymerase III was affinity chromatography using a DNA-cellulose column with stepwise additions of solutions containing increasing concentrations of KCl for elution (Figure 1C). Most of the DNA polymerase activity was eluted when the KCl concentration was increased from 200 to 500 mM, whereas most of the protein either did not bind to the column or was eluted in the presence of 200 mM KCl. Fractions corresponding to the peak of activity were stored in liquid nitrogen where activity was stable for at least 6 months.

Sedimentation Velocity. The final step in the purification of DPase III was rate-zonal centrifugation. The enzyme was found to possess activity with a number of template-primers in addition to poly(rA) · $(dT)_{12-18}$ (see Table II). Among these, activity was particularly high with poly(dA) · $(dT)_{12-18}$ and with poly(dC) · $(dG)_{1-18}$ in the presence of Mg^{2+}. The sedimentation rates of the DNA polymerase activities measured with these two template-primers and with poly(rA) · $(dT)_{12-18}$, Mn^{2+} were assessed using glycerol gradients containing 250 mM KCl. Results using the DPase III preparations at both the hydroxylapatite step (D3P3H1) and DNA-cellulose step (D3P3H1DC) are shown for comparison in Figure 2. In the case of the hydroxylapatite en-

Table II: Template-Primer Specificity of DNA Polymerase III.

Template (160 μg/ml)	Primer (32 μg/ml)	Deoxynucleotides		Amount of ^{3}H-Labeled Deoxynucleotide Incorporated per Reaction[a] (Δpmoles × 10^2)	
				Divalent Cation	
		^{3}H-labeled (1.3 μM)	Unlabeled (1.3 μM)	Mn^{2+} (0.5 mM)	Mg^{2+} (4 mM)
None	None	dTTP	None	0	0
Poly(rA)	None	dTTP	None	0	0
None	$(dT)_{12-18}$	dTTP	None	0	0
Poly(rA)	$(dT)_{12-18}$	dCTP	None	0	0
Poly(rA)	$(dT)_{12-18}$	dTTP	None	67	15
Poly(rA)	Poly(dT)	dTTP	None	66	21
Poly(rA)	$(rU)_{10}$	dTTP	None	33.6	0
Poly(rA)	Poly(rU)	dTTP	None	2.1	0
Poly(rA)	$(dT)_{8-3'OAC}$	dTTP	None		0.5
Poly(dA)	$(dT)_{12-18}$	dTTP	None	28.6	56
Poly(dA)	Poly(dT)	dTTP	None	3	180
Poly(dA)	$(rU)_{10}$	dTTP	None	0	0.2
Poly(dA)	Poly(rU)	dTTP	None	0	0
Poly(dA)	$(dT)_{8-3'OAC}$	dTTP	None	0	0.3
Poly(dT)	$(dA)_{12-18}$	dATP	None	3.4	0
Poly(dT)	Poly(dA)	dATP	None	2	4
Poly(dT)	Poly(rA)	dATP	None	0.4	5.5
Poly[d(A-T)]	None	dTTP	dATP	1	6
Poly(rI)	$(dC)_{12-18}$	dCTP	None	0	0.3
Poly(rC)	$(dG)_{12-18}$	dGTP	None	0.5	0
Poly(rU)	$(dA)_{12-18}$	dATP	None	0	0
Poly(rU)	Poly(dA)	dATP	None	0	0
Poly(dC)	$(dG)_{12-18}$	dGTP	None	12	116
Poly(dC)	Poly(dG)	dGTP	None	0	0
Poly(dG)	$(dC)_{12-18}$	dCTP	None	0	0
Poly(dG)	Poly(dC)	dCTP	None	0	0
Native C.T.DNA	None	dTTP	dATP, dCTP, dGTP	0.2	2.7
Denatured C.T.DNA	None	dTTP	dATP, dCTP, dGTP	0.3	2.5
Activated C.T.DNA	None	dTTP	dATP, dCTP, dGTP	0.6	3.4
Activated S.S.DNA	None	dTTP	dATP, dCTP, dGTP		3.3
MuLV 70S RNA	None	dATP, dCTP, dGTP	dTTP	0	0
MuLV 70S RNA	$(dT)_{12-18}$	dATP, dCTP, dGTP	dTTP	0	0

[a] Reaction mixtures contained 3 ng of DPase III protein (D3P3H1DC) and were performed as described under Methods except that they contained the divalent cation, template-primer, and deoxynucleoside 5′-triphosphate(s) indicated.

zyme preparation, congruent peaks of activity sedimenting faster than the bovine plasma albumin marker at approximately 8 S were observed under the three reaction conditions. Two additional peaks that sedimented at 5.8 S and 2.6 S were observed using poly(rA) · $(dT)_{12-18}$, Mn^{2+}. The fractions containing the 5.8 S peak of poly(rA) · $(dT)_{12-1}$, and Mn^{2+} activity were also active with poly(dA) · $(dT)_{12-18}$, Mg^{2+}. In the case of the DNA-cellulose enzyme preparation, a single peak was observed sedimenting at approximately 8 S when the gradient fractions were assayed under each of the reaction conditions (Figure 2B); the 2.6 S peak was no longer present, and a small shoulder of poly(rA) · $(dT)_{12-18}$, Mn^{2+} dependent activity was observed sedimenting at approximately 5.6 S. It should be noted that the 8 S peaks in the hydroxylapatite and DNA-cellulose enzyme preparations exhibited the same relative ratios of activity under the three types of reaction conditions. Fractions 5-8 corresponding to the peak of activities shown in Figure 2B were pooled, and represent the final DPase III preparation.

Analysis for Other Enzyme Activities. The DNA-cellulose preparation of DPase III was tested for activity of enzymes other than DNA polymerase using the reaction conditions described under Methods. In experiments not shown, no enzyme-dependent nuclease activity against DNA or single- and double-stranded RNA, nucleoside diphosphokinase activity, or RNA polymerase activity were detected. The preparation was also devoid of detectable DNA endonuclease activity. However, the HA- and DNA-cellulose preparations possessed abundant ribonuclease H activity. This activity was dependent upon the hybrid integrity of the RNA-DNA hybrid molecule used as substrate, and was observed with both [^{3}H]poly(rA) · poly(dT) and [^{3}H]RNA-calf thymus DNA. The DNA strand of the poly(rA) · [^{3}H]poly(dT) hybrid was not digested. The hydrolytic products of the enzyme activity were oligonucleotides.

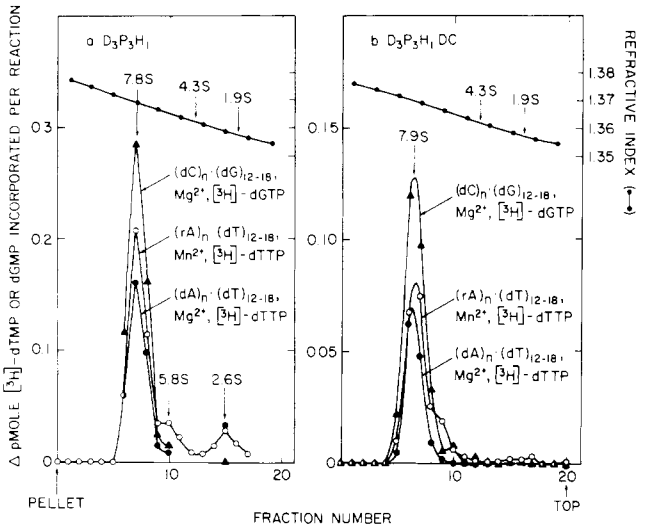

FIGURE 2. Rate-zonal sedimentation of DNA polymerase III in glycerol gradients. In panel a, 50 μl of the solution containing DNA polymerase obtained from hydroxylapatite column chromatography (D3P3H1) was diluted fourfold with 50 mM Tris-HCl (pH 7.7), 250 mM KCl, 1 mM DTT, and 0.1 mM EDTA and layered over a 4.8-ml 10-30% (v/v) linear glycerol gradient containing 50 mM Tris-HCl (pH 7.7), 250 mM KCl, 1 mM DTT, and 0.1 mM EDTA. In panel b, 50 μl of the solution containing DNA polymerase III obtained from DNA-cellulose column chromatography was diluted and layered over a gradient as in panel a. The gradients were centrifuged for 15 hr at 1° at 60,000 rpm in a SW 65 rotor. Fractions of approximately 0.25 ml were collected from below; 10-μl portions of fractions were used for assay of DNA polymerase activity as described under Methods, except that the final reaction volume was 50 μl and, where indicated, reactions contained 4 mM magnesium acetate, 160 μg/ml of poly(dC), 32 μg/ml of $(dG)_{12-18}$, and 1.3 μM [^{3}H]dGTP (27,000 dpm/pmol). Sedimentation markers, 1 mg of egg-white lysozyme (1.9 S) and 30 μg of ^{125}I-labeled bovine plasma albumin (4.3 S), were centrifuged in a companion tube; their positions in the gradients are indicated by arrows. Sedimentation coefficients of the polymerase activities were calculated relative to bovine plasma albumin at 4.3 S with the assumption that migration rate and sedimentation coefficient were linearily related.

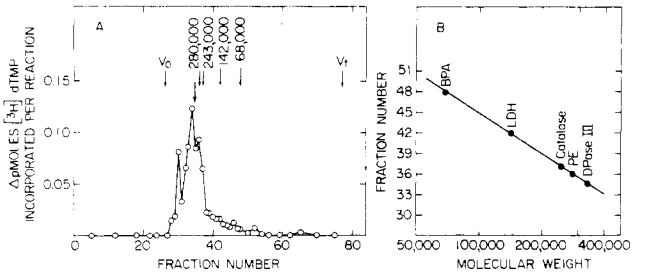

FIGURE 3: Determination of the molecular weight of DPase III by gel filtration column chromatography on Sephadex G-200. Two columns (0.9 × 60 cm) arranged in sequence were packed with Sephadex G-200 and equilibrated with 50 mM Tris-HCl (pH 7.0), 10% glycerol, 250 mM KCl, 1 mM DTT, and 0.2 mM EDTA; 200 μl of DPase III (D3P3H1DC) dissolved in the same solution was pumped onto the bottom of the first column, and the columns were developed at 4° using upward flow at a flow rate of 6 ml/hr. Each fraction contained approximately 1 ml. DNA polymerase activity was measured as described under Methods using 30-μl portions of fractions and poly(rA)·$(dT)_{12-18}$,Mn^{2+}; 7% of the enzyme activity applied to the column was recovered. The void volume (V_0) and exclusion volume (V_t) were determined using Blue Dextran 2,000 and free ^{125}I, respectively; smaller arrows in panel A indicate the elution positions of the marker proteins: molecular weight 280,000, B-phycoerythrin (PE); 243,000, catalase; 140,000, lactate dehydrogenase (LDH); 68,000, [^{125}I]bovine plasma albumin (BPA). Calculation of the molecular weight of DPase III (panel B) was performed according to the method described by Andrews (1964) using the migration position indicated by the larger arrow in panel A.

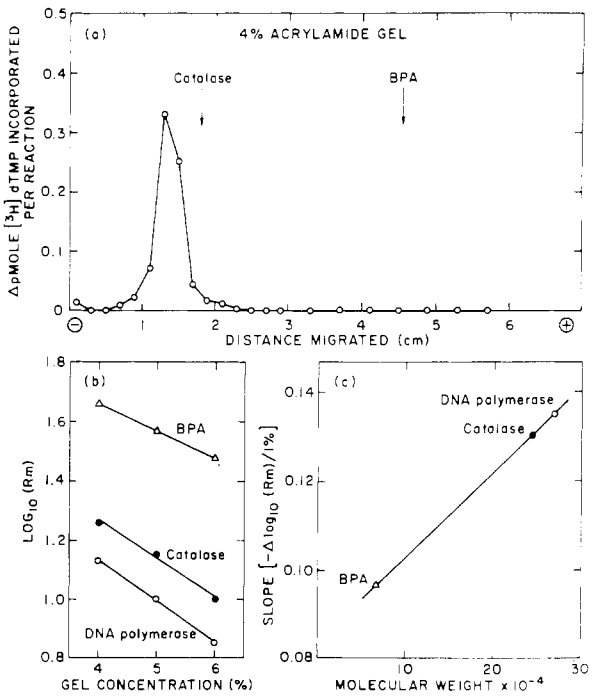

FIGURE 4: Determination of the molecular weight of DNA polymerase III by polyacrylamide gel electrophoresis. Electrophoresis under nondissociating conditions using 4, 5, and 6% acrylamide gels and assays for DNA polymerase activity with poly(rA)·$(dT)_{12-18}$,Mn^{2+} were performed as described under Methods. The figure shows (a) an electrophoretogram of the 4% acrylamide gel, (b) the relative migrations of the proteins in the gels of different acrylamide concentrations, and (c) the calculation of polymerase molecular weight (Hedrick and Smith, 1968). Recoveries of enzyme activity applied to the gels ranged between 70 and 86%. Molecular weights of the marker proteins, catalase and BPA, were taken as 243,000 and 68,000, respectively.

The ribonuclease H and DNA polymerase activities did not precisely copurify during the DNA-cellulose step of the purification procedure and the two activities did not cosediment during the final gradient centrifugation step. The ribonuclease H activity sedimented at approximately 3 S, and was completely separated from the DPase III activity.

Molecular Weight Determinations and Physical Properties of DPase III. The sedimentation coefficient of the main peak of DPase III was 7.9 S,[5] relative to bovine plasma albumin at 4.3 S. This is a value expected for an average globular protein with a molecular weight of approximately 165,000 (Smith, 1970). However, analysis of catalase in the glycerol gradient system shown in Figure 2 revealed that it sedimented only slightly faster than DPase III at approximately 8.5 S. Based upon the molecular weight of catalase (243,000) and its sedimentation behavior in this system, the molecular weight of DPase III was calculated to be approximately 230,000. The molecular weight of DPase III was also investigated by gel filtration column chromatography using Sephadex G-200, and by polyacrylamide gel electrophoresis under nondissociating conditions. In gel filtration, migration of the enzyme activity was heterodisperse, but the major portion of the activity migrated as a globular protein of molecular weight 315,000 (Figure 3). Electrophoresis at pH 7.5 in 4% acrylamide gels (Figure 4a) revealed that the enzyme activity migrated in a single band. These and similar data obtained using 5 and 6% gels at pH

[5] The average of eight values obtained in separate experiments; the range was 7.4-8.2.

7.5 (Figure 4b) indicated a molecular weight of approximately 270,000 (Figure 4c).

Since DPase III is a relatively high molecular weight DNA polymerase, we attempted to dissociate the enzyme into lower molecular weight species possessing activity. The sedimentation pattern of the enzyme was not changed by centrifugation in gradients containing 500 mM KCl and 0.2% Tween-80 or by treatment with a combination of 60 μg/ml of RNase B and 6 μg/ml of DNase I at 37° for 30 min prior to centrifugation in the usual glycerol gradient containing 250 mM KCl. Treatment of the enzyme with 125 mM ammonium sulfate (Hecht and Davidson, 1973) prior to centrifugation also did not result in dissociation, but resulted in the formation of an aggregate representing 30% of the activity that sedimented at approximately 11 S. All of the 7.9 S form of DPase III aggregated into an 11 S form when the enzyme was sedimented in gradients containing 50 mM instead of 250 mM KCl.

The isoelectric point of DPase III was also examined. The enzyme activity measured with poly(rA) · $(dT)_{12-18}$ and Mn^{2+} was recovered in a broad peak between pH 6.2 and pH 4.8, with the highest value at approximately pH 5.8. This isoelectrofocus behavior was similar to that of MOPC-104E DPase I (pH 5.4), but was different from those of MOPC-104E DPase II and the MuLV DPase which electrofocused at pH 9.4 and pH 6.5, respectively (Wilson, Matsukage and Papas, in preparation).

Product Identification. The radioactive product from reactions containing poly(rA) · $(dT)_{12-18}$ and Mn^{2+} possessed chemical properties characteristic of DNA; it was rendered cold acid-soluble by treatment with both pancreatic DNase I and 0.6 N perchloric acid at 72°, but not by treatment with 0.5 N NaOH, RNase B, or trypsin. In other experiments, incubations were performed using [^{32}P]dTTP labeled in the α position as substrate instead of [^{3}H]dTTP. The radioactive reaction product was then isolated and nearest neighbor analysis was performed; the product was treated first with micrococcal nuclease and then with spleen phosphodiesterase in a manner known to digest DNA into 3′-deoxymononucleotides (Josse and Swartz, 1963). Thin-layer chromatographic analysis (Ross *et al.*, 1973) of this digested material revealed that more than 90% of the radioactivity recovered cochromatographed with authentic 3′-dTMP.

The size of the ^{3}H-labeled reaction products formed in the usual reaction containing poly(rA) · $(dT)_{12-18}$, Mn^{2+} were investigated in the experiment shown in Figure 5. Products in a reaction mixture after 60 min of incubation were isolated, and treated first with 0.1% SDS and then with 0.3 N NaOH for 16 hr at 37°. The solution was then neutralized and subjected to sucrose gradient analysis at pH 7.7. Most of the radioactive product sedimented as a sharp peak at approximately 15 S. This sedimentation rate was similar to that found for the sample of poly(rA) used as template (Bohn and Wilson, 1974). The $(dT)_{12-18}$ used as primer sedimented at 1 S–2 S.

The question of whether the radioactive poly(dT) produced during the incubation could be recovered in hybrid form with poly(rA) was investigated by determining (1) the sensitivity of the radioactive product to digestion by *Aspergillus* nuclease S1, and (2) the density of the product by centrifugation to equilibrium in a cesium sulfate gradient. More than 75% of the reaction product was resistant to nuclease S1 when the digestion was performed at 50°, a temperature at which a poly(rA) · poly(dT) hybrid would have been partially melted. Most of the radioactive product was digested by the S1 nuclease after it was heated at 100° for 2 min and then rapidly chilled in the presence of unlabeled poly(dT). The radioactive reaction product was found to band at a density of 1.438 g/ml in a cesium sulfate gradient (pH 7.5). This value was identical with the density of a 1:1 poly(rA) · poly(dT) hybrid, and differed slightly from the densities of T7 DNA and poly(dT).

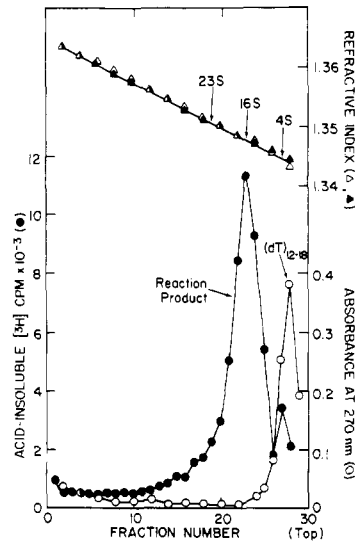

FIGURE 5: A composite graph showing rate-zonal sedimentation of the oligo(dT) primer and the base-treated radioactive products formed in a reaction containing poly(rA) · $(dT)_{12-18}$ and Mn^{2+}. A reaction mixture contained the components described under Methods and 0.25 ng of DNA polymerase III protein (D3P3H1DC). After incubation, the reaction mixture was adjusted to 0.5% in SDS, and then subjected to column chromatography on Sephadex G-25 to remove the unincorporated substrate; the radioactive material in the void volume was adjusted to 0.3 N in NaOH, incubated at 60° for 16 hr, and neutralized by addition of HCl. A portion of the solution was then layered over a 5-ml 5–20% linear sucrose gradient containing 50 mM Tris-HCl (pH 7.7), 100 mM NaCl, and 1 mM EDTA, and centrifuged for 2 hr at 45,000 rpm in a SW 50.1 rotor at 5°. Gradients were fractionated from below; 50 μg of $(dT)_{12-18}$ and [^{3}H]uracil labeled *E. coli* 23 S, 16 S, and 4 S RNA were centrifuged in separate companion tubes. The positions of the RNA markers are indicated by the arrows. The open symbols indicate the refractive index and absorbance for the gradient containing $(dT)_{12-18}$; solid symbols indicate the refractive index and cold acid-insoluble radioactivity for the gradient containing the reaction products.

Template Specificity

The template specificity of DPase III was examined at one concentration of template and primer under the conditions of the usual reactions containing either 0.5 mM Mn^{2+} or 4 mM Mg^{2+} (Table II). In the presence of either divalent cation, no significant incorporation was observed in reactions without template-primer or in reactions containing either template or primer alone. Incorporation was not observed in reactions containing combinations of template and primer that were not complementary for base pairing, when the deoxynucleotide was not complementary with the template, or when the 3′-hydroxyl group of the primer was blocked with an acetyl group. The relative activities in the presence of the various template-primers were different in reactions containing the two divalent cations.

In reactions containing 0.5 mM Mn^{2+}, the amount of deoxynucleotide incorporated was similar in the presence of poly(rA) · $(dT)_{12-18}$ and poly(rA) · poly(dT). This level of incorporation was much higher than in reactions containing poly(rA) · poly(rU). Poly(dA) · $(dT)_{12-18}$ was 42% as effec-

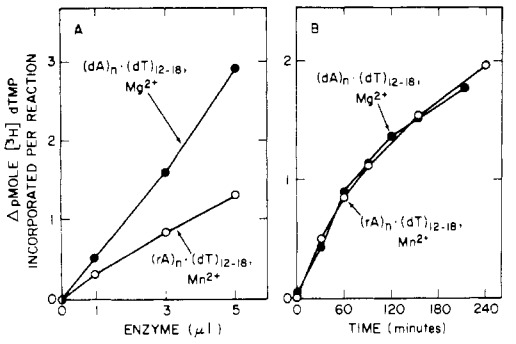

FIGURE 6: The effects of the amount of enzyme (a) and the time of incubation (b) upon the activity of DNA polymerase III. Reactions were performed as described under Methods; in panel A 1 μl of the enzyme preparation (D3P3H1DC) contained 1 and 3 ng of protein, respectively, for the poly(rA) and poly(dA) containing reactions, whereas each reaction in panel B contained 3 ng of protein (D3P3H1DC).

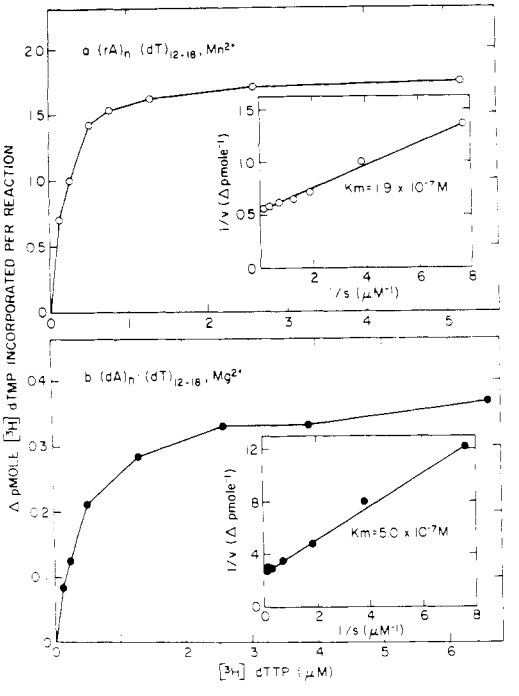

FIGURE 7: The effect of dTTP concentration upon the activity of DNA polymerase III. Reactions were performed as described under Methods, except for the indicated concentrations of dTTP; in panels a and b reaction mixtures contained 5 and 3 ng, respectively, of DNA polymerase III protein (D3P3H1DC).

tive as poly(rA) · $(dT)_{12-18}$ for promoting dTMP incorporation, and incorporation of dAMP in the presence of poly(dA) · poly(dT) was much lower than incorporation in reactions containing poly(dT) · $(dA)_{12-18}$. Deoxynucleotide incorporation in the presence of the other template-primers tested was much lower.

In reactions containing 4 mM Mg^{2+}, deoxynucleotide incorporation was highest in the presence of poly(dC) · $(dG)_{12-18}$ and poly(dA) · poly(dT); much less incorporation was observed in the presence of poly(rA) · poly(dT). Incorporation of dAMP was approximately 30 times lower than dTMP incorporation in reactions containing poly[d(A-T)] or poly(dA) · poly(dT). Relatively low levels of incorporation were observed in reactions containing activated, native, and denatured calf thymus or salmon sperm DNA. MuLV 70 S RNA was not effective as template.

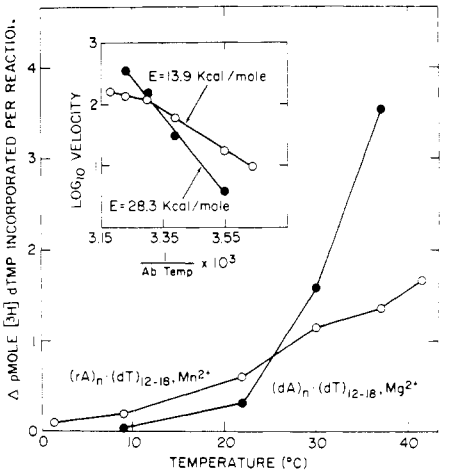

FIGURE 8: The effect of temperature upon the activity of DNA polymerase III. Reactions were performed as described under Methods, except for the indicated incubation temperature. Each reaction contained 3 ng of DNA polymerase III protein (D3P3H1DC).

Reaction Properties of the DNA Polymerase Activity

Since the activity of DPase III was relatively high in reactions containing poly(rA) · $(dT)_{12-18}$, Mn^{2+} or poly(dA) · $(dT)_{12-18}$, Mg^{2+}, properties of and requirements for the oligo(dT) deoxynucleotidyltransferase reaction under both conditions were investigated. The purpose of these experiments was to establish reaction properties and also to examine whether the mechanism of enzyme action under the two types of conditions is identical.

Dependence upon the Time of Incubation and the Amount of Enzyme. DNA polymerase activity in the presence of both poly(rA) · $(dT)_{12-18}$, Mn^{2+} and poly(dA) · $(dT)_{12-18}$, Mg^{2+} was proportional to the amount of enzyme protein present in reactions (Figure 6A). The rate of DNA polymerase activity in both types of reactions was nearly constant during the first 60 min of incubation (Figure 6B). During the period of 60–240 min of incubation, the rate of activity in both types of reactions gradually decreased. This decrease in rate may have been due to utilization of the substrate [^{3}H]dTTP (see Figure 7), since a higher concentration of [^{3}H]dTTP (13 μM instead of 1.3 μM) resulted in linear rates of incorporation for at least 180 min. In the ensuing experiments, all nucleotide incorporations represent linear initial velocity measurements.

Dependence upon Substrate Concentration. DNA polymerase activity in the presence of both poly(rA) · $(dT)_{12-18}$, Mn^{2+} and poly(dA) · $(dT)_{12-18}$, Mg^{2+} increased hyperbolically with increasing concentration of dTTP (Figure 7). Analysis of these data using Lineweaver and Burk (1934) plots revealed linear relationships between product formation and substrate concentration. The Michaelis constants in both types of reactions were similar and were in the range of 2–5 × 10^{-7} M.

Dependence upon Temperature of Incubation. DNA polymerase activity under reaction conditions of both types increased as the temperature of incubation was increased (Figure 8). Analysis of the data using an Arrhenius plot revealed a linear relationship over the range of temperature tested for the poly(dA) · $(dT)_{12-18}$, Mg^{2+} activity, and an activation energy of 28.3 kcal/mol. However, a biphasic relationship was observed for the poly(rA) · $(dT)_{12-18}$, Mn^{2+} activity; between the temperatures of 1 and 30° the activation energy was 13.9 kcal/mol whereas, between 30 and 42°, the activation energy was lower.

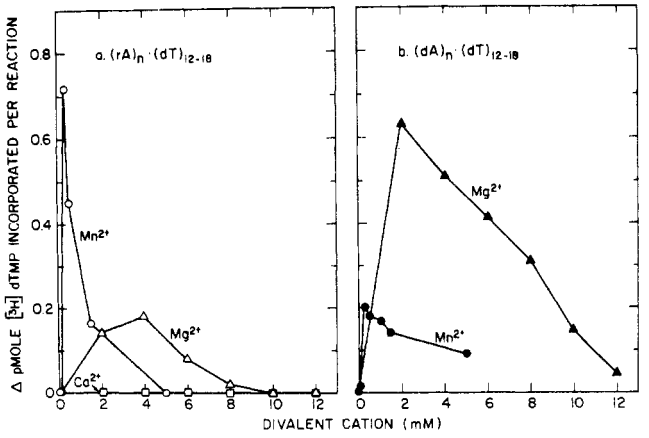

FIGURE 9: The effect of divalent cations upon the activity of DNA polymerase III. Reactions were performed as described under Methods, except for the indicated concentrations of divalent cations which were present as the following salts: $MnCl_2$, magnesium acetate, and $CaCl_2$. In panels a and b, reaction mixtures contained 2 and 6 ng of enzyme protein (D3P3H1DC), respectively. Concentrations of Mn^{2+} that were less than 2 mM were as follows: (a) 0.1, 0.3, 0.5, and 1.5 mM; (b) 0.1, 0.3, 0.5, 1, and 1.5 mM.

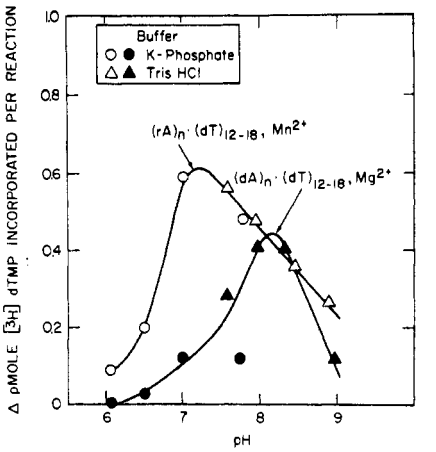

FIGURE 10: The effects of reaction mixture pH upon the activity of DNA polymerase III. Reactions were as described under Methods, except that 50 mM potassium phosphate buffer (circles) or 50 mM Tris-HCl buffer (triangles) was used, as indicated. Each reaction contained 1.5 ng of enzyme protein (D3P3H1DC). pH was determined in 0.4-ml reaction mixtures that were identical with the usual 25-μl reactions, except that [^{3}H]dTTP was substituted with unlabeled dTTP and reaction mixtures contained no enzyme protein.

Dependence upon Divalent Cations. DNA polymerase activity with poly(rA) · $(dT)_{12-18}$ (Figure 9a) and poly(dA) · $(dT)_{12-18}$ (Figure 9b) was dependent upon the presence of a divalent cation in the reaction mixture. The poly(rA) · $(dT)_{12-18}$-dependent activity was highest in the presence of 0.5 mM Mn^{2+} and no activity was observed in reactions containing Ca^{2+}. The poly(dA) · $(dT)_{12-18}$-dependent activity was highest in the presence of 2 mM Mg^{2+}. In reactions of both types, activities were inhibited by higher concentrations of divalent cations.

Dependence upon Reaction Mixture pH. DNA polymerase activity in the presence of poly(rA) · $(dT)_{12-18}$, Mn^{2+} was optimal between pH 7.0 and 7.6, whereas in the presence of poly(dA) · $(dT)_{12-18}$, Mg^{2+} it was optimal between pH 7.9 and 8.3 (Figure 10).

Dependence upon Concentration of Monovalent Cation. The effect upon the poly(rA) · $(dT)_{12-18}$, Mn^{2+} activity of the chloride salts of potassium, sodium, ammonium, and lithium is shown in Figure 11a. Enzyme activity was relatively low in the presence of lithium; varying the concentrations of the other three cations revealed bimodal responses in the enzyme activity. This was particularly marked with potassium and ammonium where the first peak of activity occurred at 90 mM and the second peak between 175 and 250 mM; with sodium a prominent peak was observed between 90 and 150 mM and a shoulder between 250 and 300 mM. In the case of the poly(dA) · $(dT)_{12-18}$,Mg^{2+} activity, the effect of KCl was tested (Figure 11b). The enzyme activity was highest in the presence of 50 mM KCl and was inhibited as the KCl concentration was increased.

Inhibitors and Reaction Requirements. DNA polymerase activity with both poly(rA) · $(dT)_{12-18}$,Mn^{2+} and poly(dA) · $(dT)_{12-18}$,Mg^{2+} was inhibited 60–80% by 1 mM *N*-ethylmaleimide and completely by 1 mM *p*-hydroxymercuribenzoate (Table III). Activity of both types was not inhibited by 0.5 mM spermidine, but was slightly inhibited by 1 mM pyridoxal phosphate and was inhibited by 1 mM sodium pyrophosphate and by 1 mM ATP; deoxythymidine diphosphate was not incorporated. Activity under both conditions was much more sensitive to *o*-phenanthroline than to *m*-phenanthroline. However, a higher concentration of *o*-phenanthroline was required for complete inhibition of the

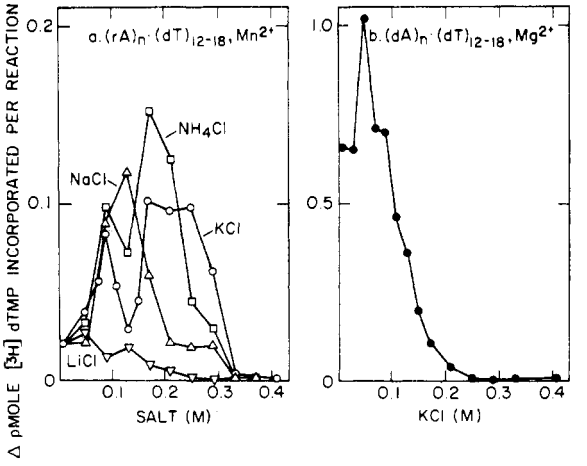

FIGURE 11: The effects of monovalent cations upon the activity of DNA polymerase III. Reactions containing 0.8 ng (a) and 3 ng (b) of enzyme protein (D3P3H1DC) were as described under Methods, except that they contained the indicated final concentration of salt including 10 mM KCl which was contributed by the enzyme solution.

poly(dA) · $(dT)_{12-18}$,Mg^{2+}-dependent activity. Antiserum capable of inhibiting mouse oncornavirus-associated DNA polymerase did not inhibit.

Stability of the DPase III Activity. The effect of incubation of the enzyme at 42° in the presence of the usual reaction components except for the template-primer and substrate was investigated. After 60 min of incubation at 42° the activities measured with poly(rA) · $(dT)_{12-18}$,Mn^{2+}, poly(dA) · $(dT)_{12-18}$,Mg^{2+}, and poly(dC) · $(dG)_{12-18}$,Mg^{2+} were markedly reduced. Analysis of these data using semi-log plots revealed linear relationships between loss of activity and time of incubation. With poly(rA) or poly(dA) as template, the loss occurred with a half-life of 26 min whereas, with poly(dC) the half-life was 34 min. In other studies with the enzyme in a solution containing 250 mM KCl, approximately 36% of enzyme activity was lost as a result of one freezing and thawing, or allowing the enzyme preparation to stand at 0–1° for 4 hr. In contrast, the poly(rA) · $(dT)_{12-18}$,Mn^{2+}-dependent activity was completely inacti-

Table III: Properties of the Nucleotide Incorporation Reaction Promoted by DNA Polymerase III.

	Modification	Amount of [^{3}H]dTMP Incorporated per Reaction (%)	
		Reaction Condition	
		Poly(rA)·(dT)$_{12-18}$, Mn^{2+}	Poly(dA)·(dT)$_{12-18}$, Mg^{2+}
A			
1.	None[a]	100 (0.13)[b]	100 (0.03)
2.	Change DTT from 1.1 to 0.1 mM	215	80
3.	No. 2 plus 1 mM *N*-ethylmaleimide	38	21
4.	No. 2 plus 1 mM *p*-hydroxymercuribenzoate	0	0
5.	Plus 0.5 mM spermidine	114	121
6.	Plus 1 mM pyridoxal phosphate	72	51
7.	Plus 1 mM sodium pyrophosphate	23	50
8.	Plus 0.1 mM ATP	40	0
9.	Minus 1.3 μM [^{3}H]dTTP, plus 1 μM [^{3}H]TDP	0	0
10.	Plus 0.25 mM Mn^{2+}	83	
11.	No. 10 plus 0.3 mM *o*-phenanthroline[c]	1	
12.	No. 10 plus 1 mM *m*-phenanthroline	80	
13.	Plus 1 mM Mg^{2+}		123
14.	No. 13 plus 1 mM *o*-phenanthroline		28
15.	No. 13 plus 1.5 mM *o*-phenanthroline		14
16.	No. 13 plus 2 mM *o*-phenanthroline		3
17.	No. 13 plus 2 mM *m*-phenanthroline		48
B			
	None[d]	100 (0.04)	100 (0.06)
	Plus 0.3 μl of control serum	217	148
	Plus 1.0 μl of control serum	195	185
	Plus 0.3 μl of MuLV Pol. antiserum	169	238
	Plus 1.0 μl of MuLV Pol. antiserum	212[e]	187

[a] The reaction conditions were described under Methods. [b] The values in parentheses refer to the Δpmoles of [^{3}H]dTMP incorporated. [c] In experiments with phenanthrolines, the enzyme was preincubated with the inhibitor for 15–30 min at 0–1°, phenanthrolines were dissolved in 10% ethanol and the final reaction mixtures contained 0.06–0.4% ethanol; these concentrations of ethanol alone had no significant effect upon enzyme activity. [d] Reactions were performed as described under Methods except that all contained 4 mM magnesium acetate and 40 mM KCl, and the template-primer and [^{3}H]dTTP were added to reaction mixtures 2 min after addition of the serum. [e] Under this condition, activity of MuLV DNA polymerase was inhibited by 93%.

vated by holding the enzyme for 2 hr at 0–1° in 50 mM Tris-HCl (pH 7.7), 20% glycerol, 1 mM DTT, and 0.1 mM EDTA, a solution containing no KCl.

Detection of DNA Polymerase III in Mouse Liver

It was of interest to determine whether an enzyme analogous to the myeloma DPase III is present in normal tissue, and also to determine the cellular localization of the enzyme in a tissue more amenable to subcellular fractionation. Thus, DNA polymerase in adult BALB/c mouse liver was examined. Using the same procedures that had been used for purification of the myeloma DPase III, a very similar form of enzyme was detected. This liver enzyme is also designated DPase III. In the chromatographic systems used, the behavior of the MOPC-104E DPase III and that of the liver DPase III were indistinguishable; representative DNA-cellulose column chromatograms of the two enzymes are shown in Figure 12.

Distinction of Liver DPase III from Cellular DNA Polymerases I and II. As indicated above, we have considered two cellular DNA polymerases as being well recognized in mammalian tissues (see Loeb, 1974). These two enzymes were isolated from liver using established procedures, and their chromatographic and sedimentation properties were compared with those of the liver DPase III; the peak of DPase activity was eluted from the phosphocellulose column at 240 mM KP_i, whereas the peak of DPase I activity was eluted at 115 mM KP_i and the peak of DPase II activity at 280 mM KP_i. The liver DPase III sedimented in glycerol gradients (containing 500 mM KCl and 0.2% Tween-80) faster than the BSA marker at approximately 6 S–8 S, whereas, liver DPase I and DPase II sedimented at approximately 3.4 S and 5.8 S, respectively. These properties were similar to those observed with the three analogous enzymes in MOPC-104E (Matsukage *et al.*, 1974a). Since the sedimentation properties of the liver DPase I and liver DPase III were similar, these two enzymes were further distinguished using hydroxylapatite column chromatography (Figure 13). Column fractions were assayed for DNA polymerase activity with poly[d(A-T)],Mg^{2+} for detection of DPase I and with poly(rA)·(dT)$_{12-18}$,Mn^{2+} for detection of DPase III. Poly[d(A-T)] was used because DPase I was more active with this template-primer than with activated DNA. As found previously with the MOPC-104E DPase I and III (Matsukage *et al.*, 1974a), the two liver enzymes exhibited different behaviors; the peak of DPase III was eluted at 10 mM KP_i (Figure 13A), whereas the peak of DPase I was eluted at 50 mM KP_i (Figure 13B).

Subcellular Localization of the Mouse Liver DPase III. As in the case of MOPC-104E DPase III, all of the DPase

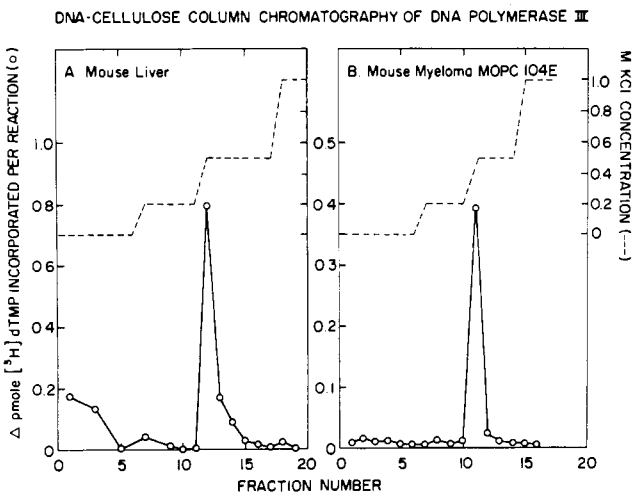

FIGURE 12: DNA-cellulose column chromatography of DNA polymerase III purified from extracts of (A) mouse liver and (B) myeloma MOPC-104E. Both DNA polymerases were purified through the hydroxylapatite column chromatography step as described in the text and in Figure 1. Preparations containing 75 and 225 μg of protein, respectively, for liver and myeloma were applied to separate 0.6 × 5 cm DNA-cellulose columns. The columns were then developed by stepwise additions of 50 mM Tris-HCl (pH 7.7), 20% glycerol, 1 mM DTT, and 0.1 mM EDTA containing increasing concentrations of KCl. DNA polymerase activity with poly(rA) · $(dT)_{12-18}$ as template-primer was determined using 3-μl portions of fractions from the liver extract chromatogram (A) and 1-μl portions from myeloma extract chromatogram (B) as described under Methods. Recovery of enzyme activity during chromatography was 20–30% in the case of each enzyme preparation.

III in the liver cytoplasmic fraction was localized in the pellet after the cytoplasmic fraction was centrifuged at 78,000*g* for 3 hr. Therefore, subcellular localization of the enzyme was different from that of liver DPase I which was localized primarily in the cytoplasmic 78,000*g* supernatant fraction. Since the 78,000*g* pellet is the expected location of mitochondrial-associated DNA polymerase(s), a more precise localization of DPase III within the crude cytoplasmic membrane fraction was undertaken. The total cytoplasmic fraction was separated into a postmitochondrial membrane fraction (the pellet after centrifugation of the 8500*g* supernatant at 78,000*g*) and a purified mitochondrial fraction. Each fraction was then extracted with solution A containing 500 mM KCl, sonicated, fractionated, and chromatographed on DEAE-cellulose columns in the usual manner for the isolation of DPase III; the "D3" fractions from the DEAE-cellulose columns were then analyzed by phosphocellulose column chromatography. The cytoplasmic high speed supernatant fraction was included in the experiment for comparison. DPase III was localized exclusively in the postmitochondrial membrane fraction (Figure 14). The purified mitochondria possessed a poly(rA)-directed activity that was not absorbed onto the phosphocellulose column (Figure 14A). However, 90% of the poly(rA) · $(dT)_{12-18}$-directed activity in the mitochondrial preparation was not extracted (data not shown). With the high speed supernatant fraction, no poly(rA) · $(dT)_{12-18}$-directed activity (Figure 14C) was detected.

Comparison of the Amounts of the DPase III Activity in Mouse Liver and MOPC-104E. The amounts of DPase III activity per gram of tissue were calculated using the results of the phosphocellulose chromatograms for the liver and MOPC-104E extracts (Table IV). Values for DPase I and DPase II are also included in Table IV for comparison. The amount of DNA polymerase III activity was approximately five times lower in liver than in the myeloma, yet its activity in liver was predominant among the three polymerases. The amounts of DPase II activity per gram of tissue were identical in the two cell types, whereas the amount of DPase I activity was more than 20 times lower in liver. Similar results have been reported on the relative activities of enzymes analogous to DPases I and II in other "stationary" and rapidly dividing tissues (See Baril *et al.*, 1973; Chang *et al.*, 1973), although the amounts of activity detected were considerably higher than those shown in Table IV.

Discussion

An enzyme in the MOPC-104E tumor termed DNA polymerase III was solubilized and then extensively purified using a series of ion-exchange chromatographic procedures, and final steps involving chromatography on DNA-cellulose and glycerol gradient centrifugation. Unfortunately, the amounts of protein recovered after the final steps in the purification were insufficient to permit careful assessment of either the degree of purity of the enzyme or its subunit composition. Analysis of the enzyme preparation after the DNA-cellulose chromatographic step by sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed six polypeptides with approximate molecular weights of 185,000, 135,000, 118,000, 66,000, 44,000, and 26,000. We do not yet know which, if any, of these polypeptides corresponds to DPase III. The highest specific activity of the DNA-cellulose preparation of the enzyme (obtained with poly(dA) · poly(dT), and Mg^{2+}) was approximately 55 nmol of [^{3}H]dTMP incorporated per min per mg of protein. Although this value is in the range of those reported for several highly purified preparations of DNA polymerase (Jovin *et al.*, 1969; Grandgenett *et al.*, 1973), it is approximately 75 times lower than values obtained for the poly(dA) · oligo(dT) and poly(rA) · oligo(dT)-dependent activities, respectively, of essentially homogeneous preparations of *E. coli* DNA polymerase I, the 3.4 S calf thymus DNA polymerase (Chang and Bollum, 1973), and a low molecular weight chicken embryo DNA polymerase (Stavrianopoulos *et al.*, 1972); the value is also much lower than the 20,000 nmoles per min per mg of protein which would be expected if DPase III is a nucleotidyltransferase with the relatively low turnover number of 10^2 molecules/sec. All of this information suggests that DNA polymerase III can be more extensively purified than in the current study.

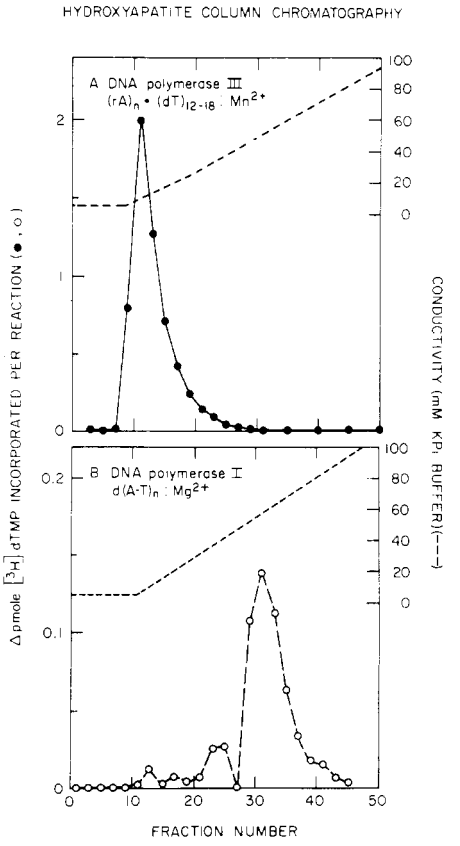

FIGURE 13: Hydroxylapatite column chromatography of mouse liver DNA polymerases I and III. Portions of the stocked enzyme preparations were dialyzed for 5 hr at 4° against 5 mM KP_i (pH 7.0), in HA solution, and then applied to separate 0.5 × 10 cm columns previously equilibrated with the HA solution containing 5 mM KP_i. In panel A, the DPase III preparation (D3P3) contained 17.3 mg of protein; chromatography and assays were performed as in Figure 1B except for the following: the column was washed with 7 ml of 5 mM KP_i in HA solution; the elution gradient was 70 ml, the flow rate was 4.2 ml/hr; each fraction contained 1.4 ml, 5 μl of fractions was tested for polymerase activity, and the overall recovery of activity was 77%. In panel B, the DNA polymerase I preparation contained 1.8 mg of protein; the column was washed with 10 ml and developed with a 35-ml gradient as described for panel A, except that the flow rate was 3 ml/hr and each fraction contained 0.8 ml. DNA polymerase activity was measured as in panel A, except that reactions contained 200 μg/ml of poly[d(A-T)], 50 mM KCl, 10 mM Mg^{2+}, 0.1 mM [*methyl*-^{3}H]dTTP (6700 dpm/pmol), and 0.5 mM each of dATP, dCTP, and dGTP; the recovery of activity was 60%.

In contrast to some DNA polymerases, the MOPC-104E DPase III possessed no detectable nucleolytic activity toward either DNA or single- and double-stranded RNA, and also possessed no detectable nucleoside diphosphokinase activity. The enzyme was not active as a DNA-dependent or messenger RNA-dependent RNA polymerase under several different reaction conditions. RNase H activity was present in some of the DPase III preparations. This activity did not precisely copurify with the DNA polymerase activity and the two activities were completely separated during the final step in the purification. This presence of RNase H could have been the result of a special affinity between the two enzymes or the partial copurification of two unassociated enzymes; there is no conclusive evidence favoring either possibility. However, RNase H, devoid of DNA polymerase activity, has been detected in a mouse myeloma (O'Cuinn *et al.*, 1973), and to the extent examined, the reaction products of the RNase H activity detected in the current study were consistent with the products expected for a polymerase-devoid cellular RNase H (Keller and Crouch, 1972).

Molecular Weight of DPase III. Results from glycerol gradient centrifugation in the presence of 250 mM KCl suggested a molecular weight for DPase III in the area of 230,000, whereas, gel filtration and gel electrophoresis under nondissociating conditions indicated molecular weights of approximately 315,000 and 270,000, respectively. It is possible that these differences in molecular weight values reflect differences in the behavior of the same form of polymerase molecule.

It is noteworthy that the rate of sedimentation in the glycerol gradient system used was not linearly related to molecular weight with proteins above 150,000. In addition to DPase III and catalase, this nonlinearity was also observed with several other proteins.

The DNA Polymerase Activities. The activity of DPase III under two types of reaction conditions was studied in order to compare properties of the enzyme reaction with different combinations of template and divalent cation. Except for the relatively low Michaelis constants for the substrate, dTTP, the requirements for maximal activity under the two reaction conditions studied, poly(rA) · $(dT)_{12-18}$, with Mn^{2+} and poly(dA) · $(dT)_{12-18}$ with Mg^{2+}, were different. Thus, the detailed mechanisms involved in the two types of reactions were not identical, yet the activities appeared to be associated with the same polymerase molecule. These differences could be due to distinct enzyme sites, or to the template–primer–divalent cation combinations alone. In any event, the results demonstrate that the detailed properties of the DPase III catalyzed reaction can vary depending upon the template–primer and divalent cation used.

DPase III in Normal Liver. The results demonstrate that adult BALB/c mouse liver contains a relatively high molecular weight, membrane-associated DNA polymerase that is active with poly(rA) · $(dT)_{12-18}$ as template–primer. This enzyme was chromatographically distinguishable from the two well-recognized DNA polymerases in liver. When the amounts of this enzyme activity were measured in various subcellular fractions of liver, the enzyme was detected in the cytoplasmic postmitochondrial membrane fraction but not in purified mitochondria or in the cytoplasmic supernatant fraction. The chromatographic behavior of this liver enzyme and that of MOPC-104E DPase III were virtually identical on DEAE-cellulose, phosphocellulose, hydroxylapatite, and DNA-cellulose. We conclude from these results that this new liver DNA polymerase and the MOPC-104E are analogous enzymes in the two cell types.

It should be noted that the liver DPase II and MOPC-104E DPase II exhibited identical chromatographic behavior and reaction properties, so far as tested. Yet, the sedimentation coefficient of the liver enzyme was 3.4 S whereas the myeloma enzyme sedimented at 2.5 S. We obtained no experimental explanation for this difference. However, it is possible that the liver enzyme could be a dimer of the 2.5 S enzyme, since the molecular weights of average globular proteins sedimenting at 2.5 S and 3.4 S are 24,000 and 40,000, respectively. Dimer formation by the chicken embryo low molecular weight DNA polymerase has been reported (Brun *et al.*, 1974).

Relationship of MOPC-104E DPase III to RNA Virus-Associated DNA Polymerases. The occurrence of DPase III in young adult BALB/c mouse liver suggests that the presence of an analogous enzyme in MOPC-104E is not the result of virus expression in the tumor. The enzyme is also

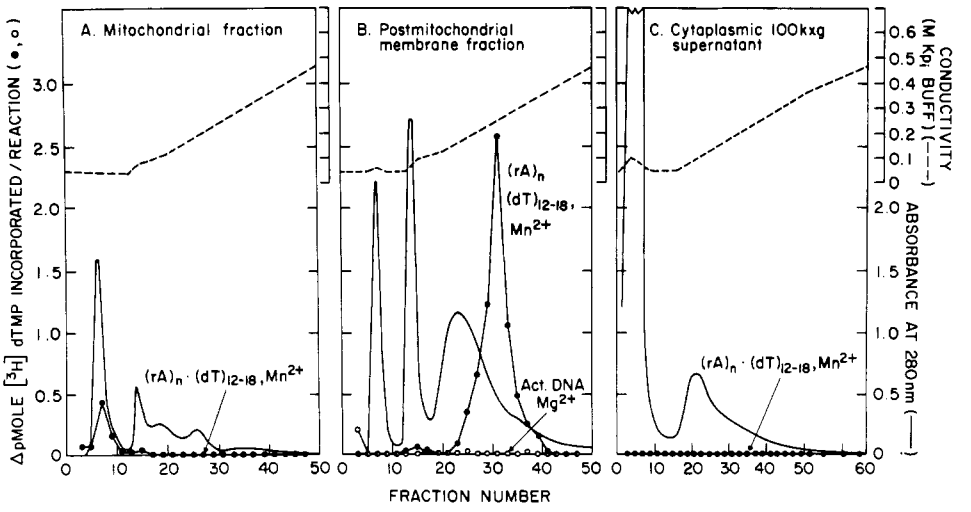

FIGURE 14: Phosphocellulose column chromatography of DEAE-cellulose fraction "D3" extracts prepared from (A) 0.8 g of purified mitochondria, (B) cytoplasmic postmitochondrial membrane fraction from 3.5 g of liver, and (C) cytoplasmic high speed supernatant fraction from 3.5 g of liver. Extracts from each subcellular fraction were prepared as described in the text, and assays for DNA polymerase activity were performed as described in Figures 1A and in 13B except that activated calf thymus DNA was substituted for poly[d(A-T)]. In panel A, 16 mg of protein was applied to a 1 × 12.5 cm column; the column was washed with 20 ml of buffer solution and eluted with a 70-ml gradient at a flow rate of 8 ml/hr. In panel B, 186 mg of protein was applied to a 1.2 × 22 cm column; the column was washed with 36 ml of buffer solution and eluted with a 175-ml gradient at a flow rate of 20 ml/hr. Total recovery of DNA polymerase III activity was 5 pmol of [^{3}H]dTMP incorporated per min per gram of liver. In panel C, 85 mg of protein was applied to a 0.8 × 10 cm column; the column was washed with 32 ml of buffer solution and eluted with a 130-ml gradient at a flow rate of 18 ml/hr.

Table IV: Comparison of the Amounts of DNA Polymerase Activities in Mouse Tissues.

Mouse Tissue	Amount of DNA Polymerase Activity Recovered[a] (units/g of Tissue)[b]		
	Polymerase		
	III	I	II
MOPC-104E Tumor	4800	900	170
Adult BALB/c liver	900	36	180

[a] The data were calculated from phosphocellulose column chromatograms (see Figure 1) performed with extracts from approximately 45 g of tissue. [b] 1 unit of activity = 1 pmol of [^{3}H]dTMP incorporated/60 min.

distinguished from RNA virus-associated DNA polymerases by the following criteria: (1) the molecular weight of DPase III is higher than those reported for mouse oncornavirus-associated DNA polymerases; (2) DPase III was more active with poly(dA) · oligo(dT) than with poly(rA) · oligo(dT) in the presence of magnesium, and was not active with either poly(rC) · oligo(dG) or poly(rI) · oligo(dC); (3) the poly(rA)-directed activity of DPase III was not inhibited by antiserum that inhibited the poly(rA)-directed activity of MuLV by more than 90%; (4) the poly(rA)-directed activity of DPase III was relatively resistant to high concentrations of monovalent cation, whereas the MuLV DNA polymerase was markedly inhibited by concentrations of KCl higher than 75 mM and was more than 90% inhibited in the presence of 200 mM KCl, the optimal concentration of DPase III activity; and (5) the K_m values of DPase III for dTTP were 10- to 100-fold lower than those of MuLV DNA polymerase. DNA polymerase III also does not appear to be identical with the DNA polymerase associated with intracisternal A-particles, since the reaction properties of the two enzymes differ markedly; DPase III was virtually inactive under the conditions for optimal activity of A-particle enzyme (Wilson *et al.*, 1974). The A-particle enzyme possessed high activity only with poly(rA) as template, and in reactions containing poly(rA) · $(dT)_{12-18}$, it differed from DPase III in divalent cation requirements and K_m value for dTTP (see Wilson and Kuff, 1972; Bohn and Wilson, 1974).

DPase III and Various Other Cellular DNA Polymerases. The evidence suggests that DPase III is a third cellular DNA polymerase, in addition to mitochondrial associated enzymes. Both because of its abundant activity and because similar experimental techniques have been used in other laboratories, it is likely that enzymes analogous to DPase III have been detected in other tissues. These enzymes include the high molecular weight R-DNA polymerase that was found in cultured mouse cells by Livingston *et al.* (1974), the human lymphocyte DNA polymerase III described by Lewis *et al.* (1974) and possibly the R-1 DNA polymerase detected in a mouse myeloma by Persico *et al.* (1973). On the basis of published information, it is not yet possible to determine whether DPase III is analogous to the mouse L-cell R-DNA polymerase, detected by Fry and Weissbach (1973). The L-cell R-DNA polymerase and the MOPC-104E DPase III appear to differ in chromatographic behavior, template specificity, apparent subcellular localization, and some requirements for maximal activity. Yet, the enzymes are similar in that both preferred Mn^{2+} over Mg^{2+} with poly(rA) · oligo(dT) as template-primer; also, the poly(rA)-directed activities of the two enzymes were relatively resistant to high concentrations of KCl, exhibited similar pH optima, and were inhibited by *p*-hydroxymercuribenzoate.

Biological Implications. Finally, some speculations about a possible *in vivo* role of DPase III seem worth mentioning. We know that (1) approximately 85% of the enzyme activity was localized in a cytoplasmic membrane fraction and (2) the activity of DPase III was relatively abundant among the polymerases in normal liver and that its activity was

only fivefold higher in the rapidly growing MOPC-104E tumor than in liver. This suggests a fairly constant cellular requirement for the enzyme, perhaps independent of genome replication. Therefore, DPase III may be involved in extranuclear synthesis of DNA that is involved in cell maintenance. While such a DNA synthesis mechanism could produce structural genes to be transcribed, it is also possible that its actions could be to specify gene expression within the cell and to transfer information between cells. For example, DNA containing specific information could be made in the cytoplasm and then transported into the nucleus where it could act to induce a precise pattern of gene expression by interacting with specific regulatory proteins. The membrane–DNA polymerase III complex may be located in the cytoplasm in order to facilitate transfer of information to surrounding cells, ultimately through DNA intermediates. Thus, cytoplasmic DNA synthesis of a specific type could be important in maintaining specialized gene expression both within the cell and among groups of cells. From an evolutionary standpoint, viruses may be an extension of an information transfer process of this type in which the viral genome happened to contain information for viral protein synthesis and assembly, and in some cases cell transformation.

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