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Multiple Forms of DNA Polymerase in Mouse Myeloma

(systematic fractionation/poly(rA)·oligo(dT) template-primer/tumors)

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ABSTRACT **Five distinct forms of DNA polymerase (deoxynucleosidetriphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7) were separated from extracts of mouse myeloma MOPC-104E using a fractionation procedure based upon sequential ion-exchange column chromatography. The enzymes were characterized according to sedimentation behavior, subcellular localization, chromatographic behavior on hydroxyapatite columns, and reaction properties. The results indicate that myeloma contains two enzymes that appear to correspond to well characterized DNA polymerases found in many other mammalian tissues, a 6S DNA polymerase localized in the cytoplasmic supernatant fraction, and a lower molecular weight (2–3S) DNA polymerase. Also present were a second 6S DNA polymerase localized exclusively in the nuclear fraction and a 6–8S DNA polymerase localized in the cytoplasmic membrane fraction. The enzyme in the cytoplasmic membrane fraction, which accounted for the predominant activity in the myeloma, was active with poly(rA)·$(dT)_{12-18}$ as template-primer, but not with activated calf thymus DNA. The detection of this distinct 6–8S membrane-bound DNA polymerase is of particular interest.**

The existence of multiple forms of DNA polymerase in eukaryotic tissues is well established (1–9). The extent of the multiplicity and nature of the enzymes involved are matters of current investigation. DNA polymerases that exhibited apparently novel template specificity were recently found in several tissues, including chicken embryo (10, 11), HeLa cells (12), rat liver (13), *Xenopus* oocyte (14), and mouse myeloma (15). A characteristic feature of these enzyme activities was that all were relatively inactive with natural DNA templates but were active with poly(rA)-containing templates; however, the relationship of these enzyme activities to previously studied DNA polymerases was not apparent. Enzymes corresponding to the well characterized 2.5–3.5S cellular DNA polymerase may have been responsible for the poly(rA)-directed activities, since this enzyme from bone marrow used poly(rA) as template with much higher efficiency than natural DNA (16). These observations prompted the current study, in which we attempted to determine the total number of both poly(rA)-directed and DNA-directed DNA polymerases in mouse myeloma (17).

METHODS AND MATERIALS

Preparations of Extract for Column Chromatography. Mouse myeloma MOPC-104E, passage 138-2, in solid tumor was excised and stored in liquid N_2 until used. All procedures were carried out at 0–5°. After the frozen tissue weighing 48 g was thawed and minced, it was suspended in 4 volumes of solution A [50 mM Tris·HCl (pH 7.7 at 5°), 1 mM dithiothreitol (DTT), and 1 mM EDTA] containing 500 mM KCl (3) and homogenized. The homogenate was sonicated in a Raytheon ultrasonic oscillator for 5 min and centrifuged at 31,500 × *g* for 30 min, and the pellet was washed with 1 volume of solution A. The wash and the supernatant were combined, dialyzed against solution A, and fractionated by ammonium sulfate precipitation; the precipitate between 25 and 65% saturation was dissolved in solution A containing 25 mM KCl and then dialyzed against 20 volumes of the same solution. The extract thus obtained was further fractionated using DEAE-cellulose column chromatography as described in Fig. 1.

Hydroxyapatite Column Chromatography. Fractions from phosphocellulose (PC) columns containing DNA polymerase activities were pooled as indicated in Fig. 1. Protein that precipitated in the presence of 65% saturated ammonium sulfate was dissolved in a solution containing 50 mM Tris·HCl (pH 7.7), 50% (v/v) glycerol, 1 mM DTT, and 500 mM-KCl and stored at −30°. A portion of this solution was dialyzed against 5 mM potassium phosphate buffer (KPi) in HA solution (500 mM KCl, 20% glycerol, 1 mM DTT, 1 mM EDTA, and 0.2% Nonidet P40), pH 6.4, and applied to a hydroxyapatite (Hypatite Clarkson Chem. Co.) column (0.8 × 15 cm); the column was developed using a 60-ml linear gradient of 5 mM to 100 mM KPi HA solution (pH 6.4–7.0).

RESULTS

Detection of Multiple Forms of DNA Polymerase. The extract prepared from whole myeloma was first separated into three fractions by DEAE-Cellulose column chromatography and then each fraction was further resolved by PC column chromatography. The separation of different forms of DNA polymerase was monitored using enzyme assays performed under three different types of reaction conditions. Several peaks of activity were observed under each type of reaction condition (Fig. 1) and the total amount of activity recovered was equal to a major portion of the DNA polymerase activity detected in the crude extract (Table 1). The position of elution from the PC column of each peak of activity was found to be reproducible to within ±10 mM KPi using extracts either from the same passage of myeloma or from a different passage of the tumor. Except in the flowthrough fractions, no common peaks of activity were observed in the PC chromatograms of DEAE-cellulose fractions D1 and D3. However, two of the

Abbreviations: PC, phosphocellulose; DTT, dithiothreitol; KPi, potassium phosphate buffer; NEM, N-ethylmaleimide; AMV, Avian myeloblastosis virus.

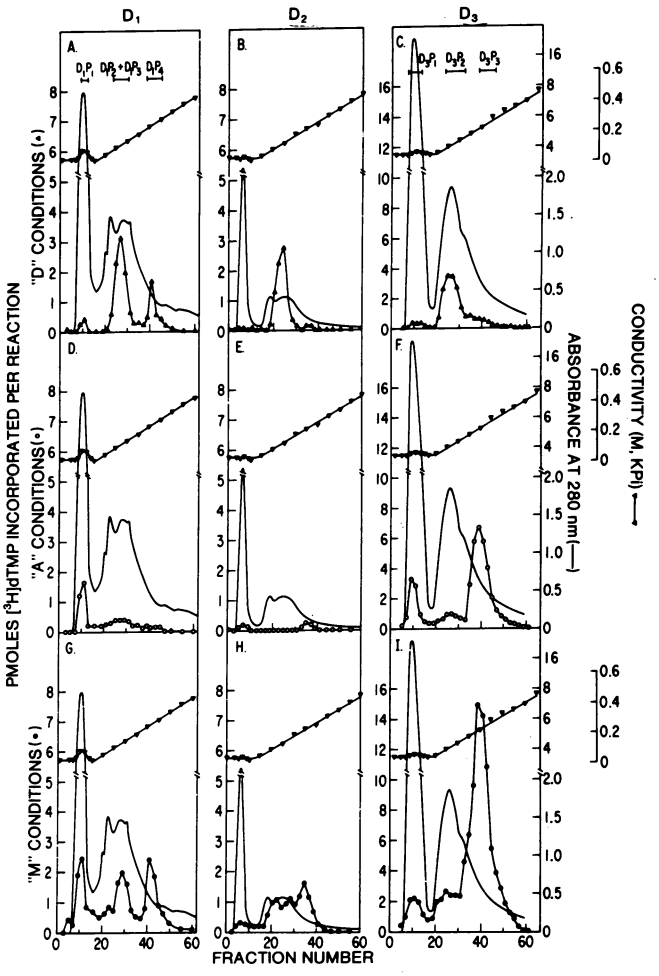

FIG. 1. Ion-exchange column chromatography on PC of DEAE-cellulose column fractions D1, D2, and D3. A myeloma extract prepared as described in *Methods and Materials* was applied to a DEAE-cellulose column. The column was washed with solution A containing 25 mM KCl and then developed by stepwise additions of solution A containing 100 mM, 250 mM, and 500 mM KCl. DNA polymerase activities were found in the flow-through fractions (termed D1 fraction) and in the fractions obtained when the KCl concentration was raised to 100 mM (termed D2 fraction) and 250 mM (termed D3 fraction). Protein in these three fractions was concentrated by ammonium sulfate precipitation, dissolved in solution B (20% glycerol and 1 mM DTT) containing 50 mM KPi (pH 6.8), dialyzed against the same solution, and then applied to PC (Sigma Chem. Co.) columns as follows: D1 fraction containing 135 mg protein was applied to a 1.4 × 20-cm column, D2 fraction containing 32 mg of protein to a 0.8 × 20-cm column, and D3 fraction containing 248 mg of protein to a 1.4 × 20-cm column. Columns were washed with 50 ml of solution B containing 50 mM KPi (pH 6.8) and developed with 220 ml of linear gradients (50–700 mM) of KPi (pH 6.8) in solution B. The flow-rate was 8 ml per hr and each fraction contained 4 ml. Conductivity was measured and expressed as KPi concentration according to the relationship between KPi and conductivity. DNA polymerase activity in 5-μl aliquots of every other fraction was assayed under "D" (Fig. 1*A*, *B*, and C), A (Fig. 1*D*, *E*, and *F*) and "M" reaction conditions (Fig. 1*G*, *H*, and *I*), as described in Table 1. Fractions containing peaks of DNA polymerase activity were pooled and designated on the basis of their relative position of elution from the two ion-exchange columns using the abbreviations D1, D2, and D3 for DEAE-cellulose peaks and P1, P2, P3, and P4 for PC peaks. For example, the enzyme activity in fraction designated D1P4 eluted as the first peak in the DEAE-cellulose chromatogram and as the 4th peak in the PC chromatogram of the material in the first DEAE-cellulose peak.

TABLE 1. *Recovery of myeloma DNA polymerase activities from cell-free extracts*

Extract	Protein/g of tumor	DNA polymerase/g of tumor		
		Reaction conditions		
		"D"	"M"	"A"
		Percent		
1. Crude Extract	100 (75 mg)	100 (25 U)*	100 (74 U)	100 (41 U)
2. 31,500 × g Supernatant	64	115	98	70
3. 31,500 × g Pellet	21	8	14	16
4. 25–65% saturated ammonium sulfate precipitate	45	88	70	74
5. DEAE-cellulose column eluate				
D1 fraction	8	88	78	26
D2 fraction	1	25	11	4
D3 fraction	15	63	42	24
6. PC column eluate				
Total in D1 fraction	5.0	50	24	7
Total in D2 fraction	0.4	31	17	0
Total in D3 fraction	4.5	57	119	83

All DNA polymerase reactions contained 50 mM Tris·HCl (pH 8.0 at 37°), 16% glycerol, 1 mM DTT, and 400 μg/ml of bovine plasma albumin. Reactions under "D" conditions also contained, in a final volume of 50 μl, 50 mM KCl; 10 mM magnesium acetate; 200 μg/ml of maximally activated calf-thymus DNA; 0.5 mM each of dATP, dCTP, and dGTP; and 0.1 mM [^{3}H]dTTP (200–700 cpm/pmol). Incubation was at 37° for 60 min. Reactions under "M" conditions also contained, in a final volume of 25 μl, 75 mM KCl, 0.5 mM $MnCl_2$, 160 μg/ml of poly(rA), 32 μg/ml of $(dT)_{14}$, and 0.5 mM [^{3}H]dTTP (220–700 cpm/pmol). Incubation was at 37° for 60 min. Reactions under "A" conditions were identical to reactions under "M" conditions, except that they contained 200 mM KCl, 160 μg/ml of $(dT)_{14}$, 1% Tween 80, and 10 mM magnesium acetate instead of $MnCl_2$. Incubation was for 75 min after a 20-min preincubation with all components except dTTP. Radioactivity converted into cold-acid-insoluble material was measured as described previously (22).

* 1 U = 1 pmol of [^{3}H]dTMP incorporated per min.

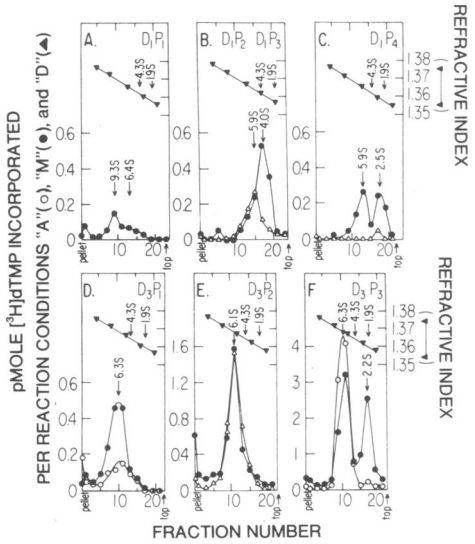

FIG. 2. Velocity sedimentation of myeloma DNA polymerase activities isolated after PC column chromatography. Fractions from PC columns containing DNA polymerase activities, as indicated in the Fig. 1, were pooled as follows: D1P1, flow-through fractions from column of D1 fraction; D1P2, fractions 24–31 from D1 fractions; D1P3, fractions 39–44 from D1 fraction; D3P1, flow-through fractions from D3; D3P2, fractions 22–29 from D3; and D3P3, fractions 37–45 from D3. Protein in 0.2 ml of each stocked fraction was precipitated with ammonium sulfate, dissolved in 0.3 ml of GG solution (50 mM Tris·HCl buffer (pH 7.7 at 5°), 500 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.2% Tween 80) and a 0.2-ml aliquot was layered over a 4.8-ml 10–30% linear glycerol gradient in GG solution. Centrifugation was in nitrocellulose tubes in a SW 50.1 rotor at 49,000 rpm for 15 hr at 2°. The gradient was fractionated from below, and the refractive index and DNA polymerase activity in 5-μl aliquots of the 200-μl fractions were assayed as described in Table 1. ^{125}I-labeled bovine plasma albumin (4.3S) and *E. coli* lysozyme (1.9S) were centrifuged in companion tubes as markers. Recovery of enzyme activities during centrifugation was greater than 70% except in the case of the activity under reaction conditions "D" in D1P4. The reproducibility of the assay for sedimentation coefficients was found to be ±0.5S.

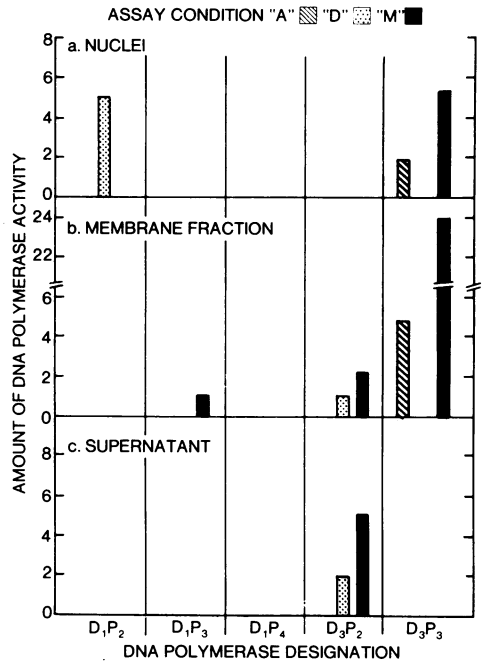

FIG. 3. Subcellular localization of mouse myeloma MOPC-104E DNA polymerase. Values determined in two similar experiments were calculated from column profiles similar to those shown in Fig. 1 and represent pmoles of [³H]dTMP incorporated per min/g of tumor. Recovery of DNA polymerases was approximately 30% of recovery from unfractionated tumor.

Freshly excised myeloma MOPC-104E, passage 140-2 (8.3 g) was minced, mixed with 33.2 ml of a solution containing 8.5% (w/v) sucrose, 10 mM Tris·HCl (pH 7.4 at 0°), 4 mM $MgCl_2$, and 1 mM DTT, and homogenized using 20 strokes in a very loosely fitting, hand operated glass-teflon homogenizer. The homogenate was centrifuged at 1,100 × *g* for 10 min and the resulting pellet was used for the preparation of sucrose-washed nuclei as follows. The pellet in a volume of 2 ml was mixed with 6 ml of a solution containing 2.0 M sucrose, 10 mM Tris·HCl (pH 7.4), 4 mM $MgCl_2$, and 1 mM DTT, and 2 ml of this mixture was layered over 2.0 ml of a solution containing 2.0 M sucrose, 10 mM Tris·HCl (pH 7.4), 4 mM $MgCl_2$, and 1 mM DTT and centrifuged for 60 min at 64,000 × *g* in an SW 50.1 rotor. The resulting pellet containing sucrose-washed nuclei was stored in liquid N_2. Nuclei were not washed with nonionic detergent because of the possibility that such washing might remove nuclear components and because the nuclei possessed no detectable cytoplasmic membrane contamination by phase contrast microscopy. The 1100 × *g* supernatant was centrifuged at 100,000 × *g* for 2 hr. The upper 90% of this supernatant was carefully withdrawn and stored in liquid N_2. The remaining supernatant was separated from the pellet and the pellet (membrane fraction) was also stored in liquid N_2.

peaks of activity that appeared in the PC chromatogram of the DEAE–cellulose fraction D2 were identical to peaks observed in DEAE–cellulose fractions D1 and D3, i.e., the last peak of activity in D2 detected under "M" and "A" reaction conditions (defined in Table 1) corresponded to the last peak of activity in D3 and the peak of activity under "D" reaction conditions (defined in Table 1) in D2 corresponded to a peak of activity in D1. The PC column fractions containing the peaks of activity were pooled, designated as shown in Fig. 1, and used in further characterization of the enzymes.

Sedimentation Properties of DNA Polymerase Activities. Sedimentation rates of the peaks of activity in the PC column eluates were investigated by centrifugation in glycerol gradients. No discrete peak of activity was detected in fraction D1P1 (Fig. 2*A*). Activities in fraction D1P2 under "D" and "M" reaction conditions sedimented at 5.9 S and 4 S, respectively (Fig. 2*B*); two peaks of activity under "M" reaction conditions sedimenting at 5.6 S and 2.5 S were observed with fraction D1P4 (Fig. 2*C*), and activity under "D" reaction conditions was present in the 2.5S peak. The relationship between these 5.6 and 2.5S activities was not further investigated. Activities in D3P1 under "A" and "M" reaction conditions sedimented at 6–8 S (Fig. 2*D*), and activities in D3P2 under both "D" and "M" reaction conditions sedimented congruently at 6.1 S (Fig. 2*E*). With fraction D3P3, activity under "M" reaction conditions sedimented at 6–8 S and 2.2 S, and activity under "A" reaction condition was found at 6–8 S only (Fig. 2*F*).

Reaction Properties of the DNA Polymerase Activities. More than 95% of the cold-acid-insoluble product produced during reaction with each of the enzymes tested was rendered cold-acid soluble after incubation with DNase (Table 2). Activities

TABLE 2. *Properties of the deoxynucleotide incorporation activities*

Modification	Enzyme designation and amount of protein per reaction: D1P2 8.2 μg	D1P3 (4S) —	D1P4 2.8 μg	D3P2 9.9 μg	D3P3 3.8 μg		*E. coli* Polymerase I 0.05 μg	AMV Polymerase 0.9 μg
	Reaction conditions*							
	"D"	"M"	"D"	"D"	"A"	"M"	"D"	"M"
	% Deoxynucleotide Incorporation per Reaction							
1. None	100 (1.3)†	100 (0.09)	100 (0.5)	100 (1.6)	100 (3.9)	100 (7.9)	100 (46.3)	100 (56.6)
2. None; (+) DNase	0.5	—§	0	2	0	0	0	—
3. (−) divalent cation	—	0	—	—	0	0	—	0
4. (−) template-primer	2	0	0	4	0	0	0	0.7
5. (−) [^{3}H]dTTP and dNTs; (+) 0.25 mM [^{3}H]dCTP	76	14	60	20	6	3	0.9	0.7
6. (−) template-primer and [^{3}H]dTTP; (+) [^{3}H]dCTP and oligo(dT)	12	0	7	4	—	—	—	—
1. 0–0.2 mM DTT‡	100 (1.1)	100 (0.09)	100 (0.9)	100 (2.3)	100 (4.6)	100 (7.7)	100 (60.6)	100 (48.6)
2. (+) 1 mM NEM	65	51	133	14	11	124	101	10

Reactions contained the components indicated in the table and were performed as described in Table 1, except for fraction D1P3 in which the [^{3}H]dNT specific activity was 4,400 cpm/pmol. Incubation with 0.5 mg/ml of pancreatic DNase was for 60 min after the usual incubation. Enzyme designations refer to the PC column elution patterns shown in Fig. 1. PC fractions pooled for each enzyme preparation were as follows: for D1P2, 27, 28, and 29; for D1P4, 41, 40, and 42; for D3P2, 24, 25, and 26; and for D3P3, 37, 38, 39, and 40. The fraction designated as D1P3 was glycerol gradient fraction 19 (Fig. 2*B*). *E. coli* DNA polymerase I, fraction 7, was from Biopolymers, Inc., and the core component of AMV was prepared as described (23).

* Reaction conditions "D," "M," and "A" are defined in Table 1.

† Number in parentheses = pmol [^{3}H]dNT incorporated minus blank determined in a reaction containing no enzyme [<0.4 pmol].

‡ For myeloma enzymes, 0.1 mM DTT for "D" reaction conditions; 0.2 mM DTT for "A", and "M" reaction conditions. No DTT present in reactions containing AMV or *E. coli* DNA polymerases.

§ Not tested.

under "A" and "M" reaction conditions were dependent upon divalent cation and were reduced at least 85 % when the template-primer was omitted or when [^{3}H]dCTP was substituted for [^{3}H]dTTP. In the presence of only one deoxynucleotide, [^{3}H]dCTP, the activities of fractions D1P2 and D1P4 under "D" reaction conditions were reduced 24% and 40%, respectively, while activity of fraction D3P2 under "D" reaction conditions was reduced 80%. Incorporation in the presence of a single deoxynucleotide has been reported with DNA polymerases from other tissues (9). This incorporation of [^{3}H]dCTP was not due to terminal transferase, since very low activity was observed in reactions containing oligo(dT) alone and [^{3}H]dCTP. In reactions containing 1 mM *N*-ethylmaleimide (NEM), DNA synthesis was not inhibited in the cases of the activity of D3P3 under "M" reaction conditions and the activities of D1P4 and *Escherichia coli* polymerase I under "D" reaction conditions. In contrast, the activity of the polymerase of avian myeloblastosis virus (AMV) and both the activity of D3P2 under "D" reaction conditions and the activity of D3P3 under "A" reaction conditions were inhibited more than 85% in the presence of NEM. The activities of D1P2 and D1P3 were inhibited 35 and 49%, respectively, in the presence of NEM.

Subcellular Localization. To determine the subcellular localization of the forms of DNA polymerase, myeloma was fractionated into nuclear, cytoplasmic membrane, and cytoplasmic soluble fractions. Extracts were prepared from each fraction and then fractionated in exactly the same manner as described for the unfractionated tissue, for which three DEAE-cellulose chromatograms and nine PC chromatograms were used. Distinct peaks of DNA polymerase activity were observed in the PC chromatograms in the same position as the peaks observed using the extract from unfractionated tissue. The results are summarized in Fig. 3. The activity of D1P2 under "D" reaction conditions was localized in the nuclei, while the 4S activity of D1P3 under "M" reaction conditions was in the cytoplasmic membranes. The activity of D3P3 under "M" reaction conditions was found in the cytoplasmic membranes and nuclei in the ratio of 10:2. The activity of D3P3 under "A" reaction conditions was present in these same two fractions. The activity corresponding to D3P2 under "D" reaction conditions was detected in the cytoplasmic supernatant from centrifugation at 100,000 × *g* and in the membranes. No information was obtained on the subcellular distribution of the activity of D1P4.

Hydroxyapaptite Column Chromatography of DNA Polymerase Activities. The relationship between the various forms of DNA polymerase was further investigated by use of hydroxyapatite column chromatography with linear gradients of KPi for elution. A single peak of activity was found in the chromatogram of each PC column fraction except fraction D1P2+D1P3, where congruent peaks of activity under "D" and "M" reaction conditions eluted before a peak of activity under "M" reaction conditions. The concentration of KPi required for elution of each enzyme from hydroxyapatite is shown in Table 3 along with a summary of other information

TABLE 3. *Summary of properties of myeloma DNA polymerases*

Designation of fraction containing DNA polymerase	Reaction conditions for detection of activity in glycerol gradient*	Sedimentation coefficient after PC step $\times 10^{-13}$ S	mM of KPi required for elution from hydroxyapatite	% Inhibition by 1 mM NEM (reaction) (conditions*)	Subcellular fraction containing enzyme activity	Probable correspondence with previously found DNA polymerases
D1P2	"D"	5.9	35	35 "D"	Nuclear† only	High-molecular-weight, nuclear DNA polymerase (8, 9, 18)
D1P3	"M"	4	45	50 "M"	Membrane‡ only	—
D1P4	"D" < "M"	2.5	35	0 "D"	(Not detected)	Low-molecular-weight DNA polymerase (7)
D3P2	"D" = "M"	6.1	50	86 "D"	Supernatant,§ membrane	High-molecular-weight, cytoplasmic DNA polymerase 24
D3P3	"A" = "M"	6–8	15	90 "A"	Nuclear, membrane	—
D3P3	"M"	2.2	Not detected	0 "M"		Similar to D1P4

* Reaction conditions "D," "M," and "A" are defined in Table 1.
† Sucrose washed nuclei prepared as described in Fig. 3.
‡ Pellet after centrifugation of cytoplasmic fraction at 100,000 × *g* for 2 hr as described in Fig. 3.
§ Supernatant after centrifugation of cytoplasmic fraction at 100,000 × *g* for 2 hr as described in Fig. 3.

about the enzymes. The 2.2S enzyme in fraction D3P3 was not recovered from the hydroxyapatite chromatogram.

DISCUSSION

The aim in this study was to define the DNA polymerase content of mouse myeloma. Application of ion-exchange chromatographic procedures and three different types of assays resulted in the separation and detection of several peaks of DNA polymerase activity in the same fractionation scheme. This fact that distinct peaks of activity were isolated along with their different reaction specificities, sedimentation rates, sensitivities to NEM, and subcellular localizations suggests that each major peak represented a distinct form of DNA polymerase. The results are summarized in Table 3. It is noteworthy that two of the enzymes were not active with natural DNA as template-primer, but were readily detected using poly(rA)·oligo(dT). No enzyme characteristic of mitochondrial DNA polymerase was detected. The 6.1S enzyme localized in the supernatant fraction and the 2.5S enzyme in fraction D1P4 appear to correspond to the high and low molecular weight DNA polymerases reported as occurring in a number of mammalian tissues. The 5.9S enzyme found in the nuclear fraction was similar in size and subcellular location to the enzyme in HeLa cells, as reported by Weissbach *et al.* (8), in KB cells, as reported by Sedwick *et al.* (9) and in rat liver, as reported by Ove *et al.* (18), and may also be similar to the nuclear membrane DNA polymerase found in calf thymus by Yoshida *et al.* (19). The 6–8S and the 4S forms of enzyme possess no clear correspondence to previously studied cellular DNA polymerases. The 4S enzyme is similar in size to the DNA polymerase derived from type-C virus (20) and may correspond to one of the enzymes reported in myeloma MOPC-21 by Persico and Gottlieb (21).

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