JOURNAL OF VIROLOGY, July 1974, p. 40-46

Vol. 14, No. 1
Printed in U.S.A.

Isolation and Characterization of RNA-Directed DNA Polymerase from a B-Type RNA Tumor Virus

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Received for publication 8 February 1974

RNA-directed DNA polymerase was isolated from milk-borne B-type murine mammary tumor virus of the RIII mouse strain. The several hundred-fold-purified enzyme sediments at 5.5 to 5.7*S* with an average molecular weight of approximately 100,000. The purified enzyme is completely template dependent and responds to RNA, DNA, and synthetic templates. Stability studies indicate differential lability dependent on the exogenous template used to monitor activity.

The isolation and characterization of RNA-directed DNA polymerases have been reported for a number of C-type RNA tumor viruses obtained from diverse hosts, including avian (6, 7, 10) reptilian (21), murine (1, 14), and primate (1) sources. That these DNA polymerases are at least in part structurally distinct has been noted from immunological studies. Some immunological relatedness was noted between murine C-type polymerase and other C-type viral polymerases from lower mammals and primates; however, little or no antigenic similarity was found between the DNA polymerases from (i) mammalian and (ii) avian C-type and (iii) mammalian B-type viruses (13, 19).

Although morphologically (3) and immunologically (12) distinct from C-type RNA tumor viruses, B-type murine viruses share a number of criteria for RNA tumor viruses. These include: (i) 60 to 70*S* RNA (17), RNA-directed DNA polymerase (4, 17, 18), and (ii) polyadenylate sequences (16). This report examines some of the biochemical and physicochemical properties of the DNA polymerase associated with B-type murine mammary tumor virus, and includes a comparison of this enzyme with the DNA polymerases found associated with C-type RNA tumor viruses.

MATERIALS AND METHODS

Virus purification. Murine mammary tumor virus (MuMTV) was purified as previously described (5) from mouse milk derived from the RIII strain.

Nucleic acids. High molecular weight RNA from RIII MuMTV was prepared by disruption (1 to 1.5 mg of protein per ml) in T.15NE buffer (0.01 M Tris-hydrochloride, 0.15 M NaCl, 0.002 M EDTA, pH 8.3) containing 0.5% sodium dodecyl sulfate (SDS) and polyvinyl sulfate (50 μg/ml; General Biochemicals) Predigested Pronase (Seravac Laboratories Ltd.) was added to a final concentration of 500 μg/ml, and the mixture was incubated at 37 C for 30 min. After two extractions with phenol/cresol (17), the aqueous layers were pooled and precipitated overnight at −20 C by the addition of 0.1 volume 4 M NaCl and 2 volumes absolute ethanol without added carrier. The precipitate was collected by centrifugation at 30,000 × *g* for 15 min, washed with buffered ethanol (T.15NE, pH 8.3, and 95% ethanol [1:2, vol/vol]), and centrifuged again, as above. The pellet was resuspended in 300 μliters of T.1NE (pH 8.3) containing 0.2% SDS, layered on a 15 to 30% RNase-free sucrose gradient (Schwarz/Mann) in T.1NE buffer (pH 8.3) containing 0.1% SDS, and centrifuged at 40,000 rpm (SW50.1) for 2 h at 20 C. Fractions were collected by dripping from the bottom, and the position of high molecular weight RNA was monitored by optical density (OD) (260 nm). The appropriate fractions were pooled, ethanol was precipitated as above, and the precipitate was centrifuged at 30,000 × *g* for 15 min. The final pellet was resuspended in 0.02 M Tris-hydrochloride (pH 7.4). These preparations had a 260/280 nm ratio ≥2.0.

Salmon sperm DNA (Calbiochem, 1 mg/ml) was activated (14) by partial digestion (15 min at 37 C) with DNase I (Worthington Biochemical Corp.; 0.04 μg/ml) in the presence of 0.05 M Tris-acetate buffer (pH 7.8) containing 0.01 M magnesium acetate. DNase I was inactivated by adding NaCl to a final concentration of 1 M and heating at 59 C for 30 min.

Poly(rC)·oligo(dG)$_{12\text{-}18}$ was obtained from Collaborative Research. Poly(rA)·poly(dT) and oligo(dT)$_{10}$ were commercial products of Miles Laboratories, Inc. and P-L Biochemicals, respectively.

Solubilization of DNA polymerase. Purified RIII MuMTV preparations were disrupted (approximately 3 mg of viral protein per ml) in 0.2 M sodium phosphate (pH 6.8), 0.01 M EDTA, 0.2% (vol/vol) β-mercaptoethanol, and 1.0% Nonidet P-40 (NP-40) (7). After mixing, the suspension was incubated for 30 min at 4 C, followed by 30 min at 37 C. Insoluble

material was removed by centrifugation at 5,000 rpm (SW41) for 15 min. Approximately 70 to 80% of the total DNA polymerase activity remained in the supernatant after the latter treatment.

Column chromatography. All operations were carried out at 4 C. The supernatant from the solubilized virus preparation was diluted 10-fold with DEAE-cellulose buffer (0.02 M Tris-hydrochloride, pH 7.2, 0.2% (vol/vol) β-mercaptoethanol, 0.2% NP-40, and 30% glycerol), and was applied to a DEAE-cellulose column (1.1 by 13 cm) (DE11, Whatman) equilibrated with the same buffer. After being washed with two column volumes of DEAE buffer, enzyme activity was eluted with DEAE buffer containing 0.4 M KCl (7) at a flow rate of approximately 30 ml/h.

DEAE-cellulose fractions positive for enzyme activity were pooled, diluted 10-fold with phosphocellulose buffer (0.01 M sodium phosphate, pH 6.8; 0.001 M EDTA; 0.2% β-mercaptoethanol; 0.2% NP-40; 30% glycerol), and applied to a phosphocellulose column (0.8 by 13 cm) (P11, Whatman) equilibrated with the same buffer. After loading, the column was washed with two column volumes of loading buffer, and the enzyme was eluted with a 60-ml 0 to 0.8 M NaCl gradient contained in the same buffer at a flow rate of 10 ml/h.

No enzyme losses were observed during loading or washing with either column fractionation technique.

DNA polymerase assays. A final volume of 50 μliters contained 62.5 mM Tris-hydrochloride (pH 8.3), 10 mM $MgCl_2$, and 12.5 mM NaCl. Poly(rC)·oligo(dG)-templated reactions contained this polymer at a final concentration of 40 μg/ml, and 12.7 μM [^{3}H]dGTP (Amersham/Searle, 8,000 counts per min per pmol). Reactions templated with "activated" salmon sperm DNA contained 40 μg of DNA per ml, 2 mM unlabeled nucleoside triphosphates (Sigma), and 10 μM [^{3}H]TTP (NEN, 7,000 counts per min per pmol). Unless otherwise specified, incubation at 37 C proceded for 30 min and was terminated by chilling on ice and by the addition of 100 μliters of yeast carrier RNA (300 μg/ml) and 100 μliters of trichloroacetic acid mix (equal volumes 100% trichloroacetic acid, saturated sodium pyrophosphate, and saturated tribasic sodium phosphate). After filtering, the samples were counted in BBOT/toluene.

Rate zonal centrifugation. Rate zonal centrifugation for the determination of molecular weight of the MuMTV DNA polymerase was performed according to the procedure of Baltimore and Smoler (2). DEAE or phosphocellulose column fractions were diluted with 0.01 M Tris-hydrochloride (pH 8.3) and applied to 4.8-ml glycerol gradients (20 to 40%, vol/vol) containing 0.05 M Tris-hydrochloride (pH 7.9), 0.1 mM EDTA, 0.1 M β-mercaptoethanol, 0.05% NP-40, and 0.5 M KCl. Centrifugation was 16 to 18 h at 4 C, 40,000 rpm (SW50.1).

The gradients were fractionated by bottom puncture and drop counting. Bovine serum albumin served as the external 4.3*S* standard.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was employed for monitoring the purification of the viral DNA polymerase, and was performed by the procedure of Wolf et al. (23). Proteins were precipitated with 5% trichloroacetic acid at 0 C. The precipitate was resuspended in 0.01 M phosphate buffer (pH 7.8) containing 1% SDS and 1% β-mercaptoethanol, and solubilized at 60 C for 30 min. After cooling, glycerol was added to a final concentration of 10%, followed by the addition of bromophenol blue as tracking dye. The samples were applied to 7.5% acrylamide gels (0.5 by 7.5 cm) and electrophoresed at room temperature for 30 min at 3 mA/gel, followed by 3.5 h at 7 mA/gel. Gels were stained with Coomassie brilliant blue R-250 (10).

Protein determinations were performed according to Lowry (20), by using bovine serum albumin as standard. To avoid interference by buffer constituents, each sample was blanked with the same buffer in which the sample was contained.

RESULTS

Purification of MuMTV polymerase. Ion exchange column chromatographic techniques used in this report were according to Faras et al. (7). The solubilized viral polymerase was applied to a DEAE-cellulose column and monitored for poly(rC)·oligo(dG) and "activated" DNA-templated DNA polymerase activity as described in Materials and Methods. Enzyme activity was eluted with loading buffer containing 0.4 M KCl, as shown in Fig. 1. When linear gradients of KCl in DEAE buffer were employed, enzyme activity eluted at approximately 0.15 to 0.2 M KCl. Recovery of enzyme activity from DEAE-cellulose ranged between 80 and 95%. No losses were observed during loading and washing.

Figure 2 illustrates the elution of poly(rC)·oligo(dG)-templated DNA polymerase activity in the presence of Mg^{2+} after dilution of the peak DEAE-cellulose fractions and application to a phosphocellulose column. The bottom panel (Fig. 2) shows the same polymer-directed synthesis in the presence of Mn^{2+}, indicating the retention of cation preference upon purification of the polymerase (1, 8). Major enzyme activity eluted with 0.4 M NaCl, employing a linear gradient. Purification and concentration of the enzyme could also be accomplished by step gradient elution, i.e., after loading, the column was washed with two column volumes of loading buffer, followed by two column volumes of loading buffer containing 0.25 M NaCl, and finally two column volumes of the same buffer containing 0.6 M NaCl. In this fashion, greater than 95% of the enzyme activity eluted with 0.6 M NaCl in a volume of 1 to 2 ml. In both types of elution, DNA polymerase activity templated with "activated DNA" (not shown) was coincident with poly(rC)·oligo(dG)-directed DNA synthesis.

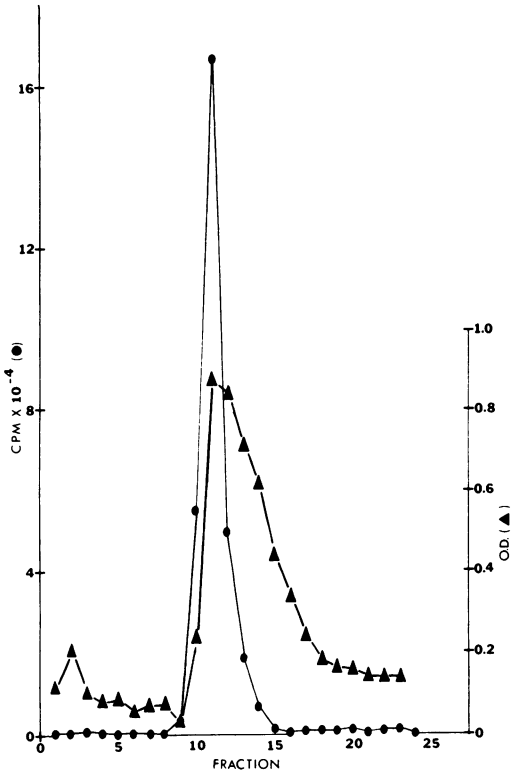

FIG. 1. *Chromatography of solubilized MuMTV DNA polymerase on DEAE-cellulose as described in Materials and Methods. Fractions (1 ml each) were collected and 5-μliter amounts were assayed for poly(rC)·oligo(dG)-templated DNA synthesis. Symbols: ▲, optical density measured at 280 nm; ●, [³H]dGMP incorporated into acid-insoluble product.*

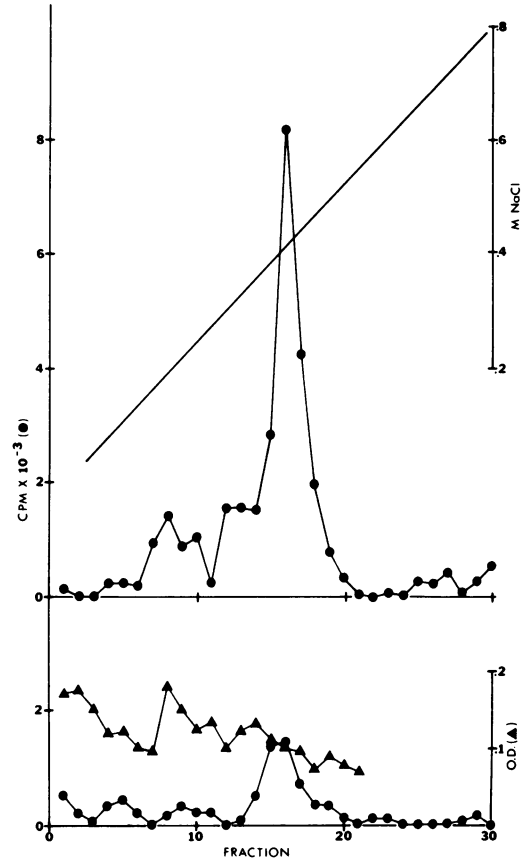

FIG. 2. *Elution pattern of MuMTV DNA polymerase on phosphocellulose. Portions (5 μliters each) were assayed from each 2-ml fraction for poly(rC)·oligo(dG)-templated DNA synthesis as described in the text. In the top panel, polymer-directed DNA synthesis in the presence of 10 mM magnesium; bottom panel, the same polymer-directed DNA synthesis in the presence of 0.4 mM manganese. Symbols: ●, [³H]dGMP incorporated into acid-insoluble product; ▲, optical density at 280 nm.*

Table 1 summarizes recoveries of enzymatic activity as a function of purification. From three separate enzyme isolations, high recoveries of activity were noted during solubilization of the enzyme and through DEAE-cellulose chromatography; however, as shown by protein determination (Table 1) and SDS-PAGE analyses (Fig. 3), no major purification was achieved. In contrast, chromatography on phosphocellulose columns effected a large degree of purification, but we consistently observed a five- to sixfold decrease in enzyme activity. The latter phenomenon was also observed if peak fractions from DEAE-cellulose were subjected to centrifugation in 20 to 40% glycerol gradients. We are investigating the possible loss of enhancing factor(s) for the DNA polymerase reaction during these purification steps. Because of some loss of recoverable enzymatic activity and the lability of the enzyme as discussed later, the determined 100-fold purification is a minimum value and probably is several times greater.

TABLE 1. *Purification of DNA polymerase from MuMTV*

Fraction	Total act[a]	Total protein (mg)	Sp act[b]	Relative purity
Solubilized virus	10,000	43.7	0.23	1
Supernatant	7,000	38.1	0.18	1
DEAE-cellulose	6,800	7.9	0.86	3.7
Phosphocellulose	1,100	<0.05	>22.0	>100

[a] Total picomoles of [³H]dGTP incorporated in 30 min by poly(rC)oligo(dG)-directed DNA synthesis.

[b] Total picomoles of [³H]dGTP incorporated in 30 min/μg of protein.

Estimation of molecular weight of DNA polymerase. As shown in Fig. 4, the DNA polymerase of MuMTV sedimented as a single component with an estimated (11) $s_{20,w}$ of 5.5*S* based on the 4.3*S* value of the external bovine

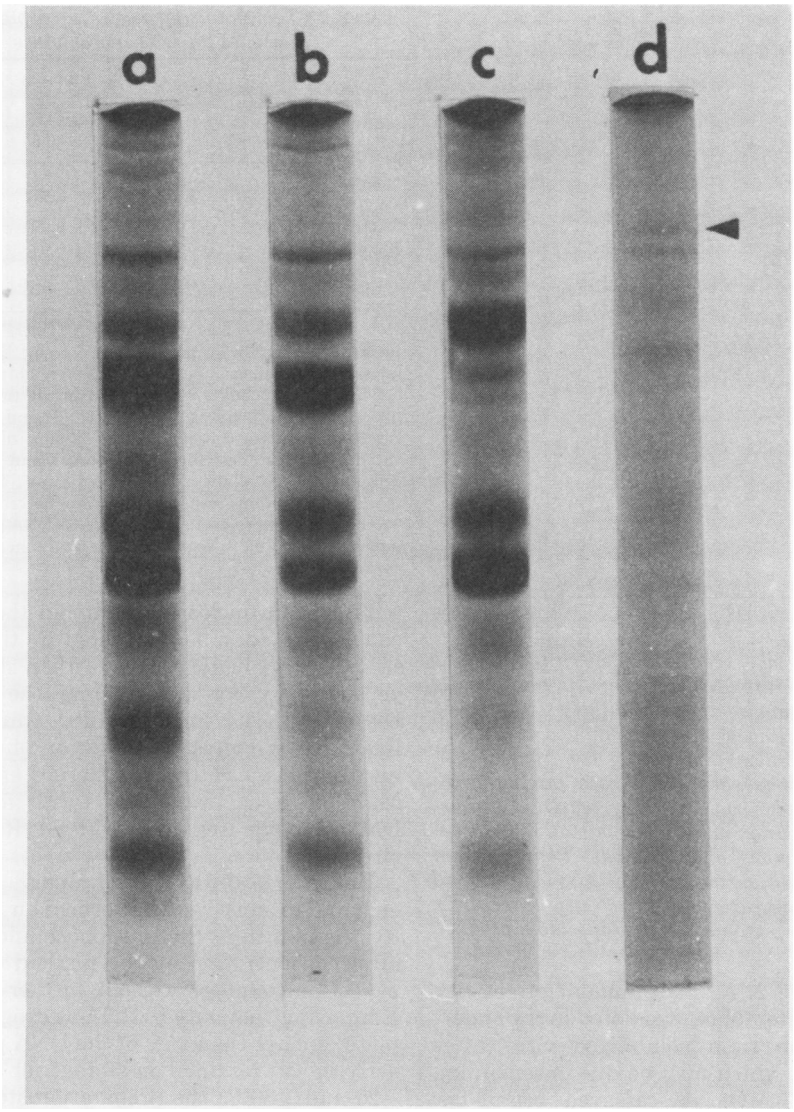

FIG. 3. *SDS-PAGE analyses of solubilized virus (a), solubilized virus after centrifugation (b), pooled peak enzyme fractions from DEAE-cellulose fractionation (c), and pooled peak enzyme fractions from phosphocellulose fractionation (d). Conditions for electrophoresis are given in the text. Protein (50 μg) was applied to gels a to c; gel d contained approximately 5 μg of protein. After electrophoresis gels were stained with Coomassie brilliant blue.*

serum albumin marker (molecular weight 68,000). From the formula $SW_1/SW_2 = (MW_1/MW_2)^{2/3}$ (15), the molecular weight of the DNA polymerase was estimated to be 98,000. The above result was obtained by centrifugation of the phosphocellulose fraction; a similar result was obtained by a similar analysis of the DEAE-cellulose peak fraction, namely, 5.7*S* with an estimated molecular weight of approximately 102,000.

SDS-PAGE analysis of the phosphocellulose peak enzyme fraction (Fig. 3d) demonstrated the enrichment of only one protein, designated by the arrow. This protein possessed a molecular weight of 100,000 and is presumably the polymerase enzyme. This was borne out of further SDS-PAGE analysis (not shown) of the peak enzyme fractions of the glycerol gradient previously described which also showed a further enrichment of the 100,000 molecular weight protein.

Template responses. DEAE and phosphocellulose fractions have a stringent requirement for exogenous template for DNA synthesis, as

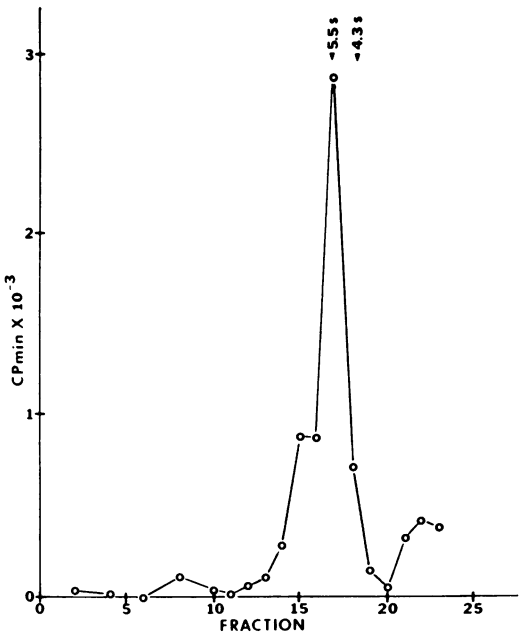

FIG. 4. *Glycerol gradient centrifugation of MuMTV polymerase. A portion of the phosphocellulose peak enzyme fraction was diluted with 0.01 M Tris-hydrochloride (pH 8.3) and applied to a 20 to 40% glycerol gradient containing 0.5 M KCl (2). Portions (10 μliters each) from each fraction were assayed for poly(rC)·oligo(dG)-directed DNA synthesis in a 2× incubation mixture (60 min at 37 C) as described in Materials and Methods. Bovine serum albumin was used as the external 4.3S marker and located by OD measurements at 280 nm. Symbol: O, [³H]dGMP incorporated into acid-insoluble product.*

shown in Table 2. A small amount of activity without added template was noted in the supernatant fraction from solubilized virus after centrifugation, which may be due to remaining viral RNA (7). Of the assayed templates, poly(rC)·oligo(dG) was found to be most efficient. Relatively less response was observed with "activated" salmon sperm DNA corresponding to template efficiencies noted by Abrell and Gallo (1) for C-type RNA tumor viruses. The decreased ratio of DNA synthesis by poly(rC)·oligo-(dG) and DNA in the phosphocellulose fraction is the result of differential lability, as discussed in the next section. MuMTV 70*S* RNA was least efficient in directing DNA synthesis, especially by the supernatant and DEAE-cellulose fractions. The relatively higher activity found for 70*S* RNA-templated DNA synthesis with the phosphocellulose fraction may be a consequence of nuclease removal at this step of purification (7). Preliminary results indicate at least a 10-fold

TABLE 2. *Template responses during purification of MuMTV DNA polymerase*

Template	Fraction		
	Supernatant	DEAE-cellulose	Phospho-cellulose
None	0.02[a]	<0.01	<0.01
Poly(rC)·oligo(dG)	3.63	7.25	17.40
"Activated" DNA	0.31	0.45	8.40
70*S* RNA	<0.01	0.01	0.60
70*S* RNA plus oligo (dT)	0.10	0.17	4.20

[a] Values expressed as picomoles of [³H]dGTP or [³H]TTP incorporated in 30 min per 15 μg of protein. The DEAE-cellulose and phosphocellulose fractions were obtained by pooling peak enzyme tubes as described in the text. The phosphocellulose fraction was concentrated (7) and stored at 4 C. Reaction mixtures containing poly(rC)oligo(dG) and "activated" DNA are described in Materials and Methods and contained 40 μg of these templates per ml. The 70*S* RNA templated reactions were performed as described for "activated" DNA and contained 8.8 μg of 70*S* RNA per ml. Where indicated, oligo(dT)$_{10}$ was added at a final concentration of 20 μg/ml.

reduction in nuclease activity in the phosphocellulose fraction as monitored by the hydrolysis of [³H]polyuridylate. Further studies will be necessary for determining the extent of DNA synthesis from purified MuMTV 70*S* RNA. Nevertheless, the addition of oligo(dT)$_{10}$ greatly enhanced the templating efficiency of MuMTV RNA, as noted (1, 22) for purified DNA polymerases from C-type RNA tumor viruses.

Enzyme stability. Preliminary studies on enzyme stability were performed on peak activity fraction from the phosphocellulose column after concentration by the method of Faras et al. (7) and storage without further treatment. Employing "activated" DNA as exogenous template, greater than 95% of the DNA polymerase activity was retained on storage for a month at −20 and −80 C; the preparation stored at 4 C lost 50% of its enzymatic activity within 11 days. In contrast, poly(rC)·oligo(dG)-templated DNA synthetic activity was found to be much more labile. A 50% decrease in enzymatic activity resulted from 5 days of storage at 4 C. Similar losses occurred after 9 and 14 days for the same preparation stored at −20 and −80 C, respectively.

Two viral DNA polymerases in RIII milk? Howk et al. (8) reported the presence of two DNA polymerases with properties of RNA-directed DNA polymerase in RIII mouse milk. These two polymerases could be distinguished on the basis of molecular weight and cation preference with the synthetic template/primer, poly(rA)·oligo(dT). The lower molecular weight polymerase preferred Mn^{2+} for poly(dT)-syn-

thesis and was inhibited by antisera prepared against mouse C-type viral polymerase. The higher molecular weight polymerase preferred Mg^{2+} for poly(dT) synthesis and was not inhibited by the above antisera. From our studies, the DNA polymerase obtained from RIII milk-borne virions prefers Mg^{2+} for optimal DNA synthesis after separation on phosphocellulose (Fig. 2) and possesses a molecular weight of approximately 100,000, clearly distinguishable from mouse C-type viral polymerase, and resembles the higher molecular weight polymerase described by Howk et al. (8) which is presumably B-type.

Because prior chromatography on DEAE-cellulose eliminates the partial separation of the two polymerases observed by Howk et al. (8) on phosphocellulose, we also attempted to detect two polymerases by assaying for cation preference after glycerol gradient centrifugation, as described above. These analyses revealed only one homogeneous peak of poly(rC)·oligo(dG)-templated polymerase activity similar to that shown in Fig. 4 with a clear preference for Mg^{2+}. We could not find an additional lower molecular weight polymerase peak requiring Mn^{2+} for optimal synthesis. Our failure to detect high levels of mouse C-type viral polymerase in our preparations may be related to variable C-type antigen expression in RIII milk (J. Charney, personal communication), and the possibility of even greater variations in C-type viral expression when the same mouse strain is housed in different laboratories.

DISCUSSION

Although the B-type viral DNA polymerase appears to be immunologically unique, its behavior on ion-exchange chromatography columns parallels that observed for the C-type viruses. As mentioned previously, chromatographic fractionation of the enzyme on phosphocellulose results in a reproducible decrease in recovery. That enhancing factor(s) have been removed is possible and studies have begun on the isolation of this factor(s).

MuMTV DNA polymerase has been determined to have a molecular weight of approximately 100,000 by glycerol gradient centrifugation and SDS-PAGE analyses. This value is clearly distinct from the molecular weights of 70,000 determined for murine and woolly monkey C-type viral DNA polymerases (1, 14), but is similar to the molecular weights (96,000 to 110,000) determined for DNA polymerases associated with the reptilian virus (21), Mason-Pfizer monkey virus (1) and avian C-type RNA tumor viruses (7, 10). Concerning the latter, the purified DNA polymerase of avian myeloblastosis virus has also been reported to be 160,000 (9) and 170,000 (1).

The differential lability for poly(rC) oligo(dG) and DNA-directed DNA synthesis observed for the MuMTV polymerase is especially interesting. Studies of isolated DNA polymerase associated with Rous sarcoma virus demonstrated that the enzymatic half-life of DNA-directed DNA synthesis was considerably longer than RNA or poly(rA)·oligo(dT)-directed synthesis (7). Recently, Marcus et al. (personal communication) conducted heat inactivation studies for a number of RNA and DNA directed DNA polymerases. For all monitored DNA polymerases, the rate of enzyme inactivation was highly template/primer dependent. In addition to considerations of enzyme conformation and interactions with template/primer and precursor, template dependent enzyme inactivation has an important practical aspect. The latter relates to the characterization of DNA polymerases by virtue of template/primer preference, and the possibility of altered preference upon storage.

ACKNOWLEDGMENTS

We thank Gary Heubel for excellent technical assistance and Janet Coccia for purification of RIII virus from milk.

This research was supported by Public Health Service contract N01-CP-33339 within the Virus Cancer Program of the National Cancer Institute, by general research support grant FR-5582 from the Division of Research Facilities and Resources, and by grant-in-aid M-43 from the State of New Jersey.

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