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An Oligonucleotide Affinity Column for RNA-Dependent DNA Polymerase from RNA Tumor Viruses

(cellular DNA polymerase/murine leukemia virus/RD-114 virus)

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ABSTRACT **Columns of $(dT)_{12-18}$-cellulose provide a one-step enrichment procedure for RNA-dependent DNA polymerase. The enzyme of the virus from RD-114 cells, as well as that from Rauscher murine leukemia virus, have been purified in this way. The preference of viral as compared to cellular DNA polymerases for $(dT)_{12-18}$ as a primer is reflected in the fact that the DNA polymerases of uninfected cells do not bind to this column. Viral enzymes have been purified and identified from crude cellular extracts.**

Affinity chromatography uses the specificity of biological macromolecules for their chemical purification (1). This technique has been applied to purification of DNA polymerases from DNA tumor viruses by Livingston *et al.* (2); who developed a selective immune adsorbent for RNA-dependent DNA polymerase from murine leukemia virus. DNA polymerases prefer a deoxyribonucleotide primer for activity. Viral polymerases show a preference for $(dT)_{12-18}$ primer as compared to $poly(dt)_{>100}$ (3); cellular polymerases prefer $poly(dT)_{>100}$ (4). We report here the purification of viral RNA-dependent DNA polymerases on a column of $(dT)_{12-18}$ covalently coupled to cellulose. Cellular DNA polymerases can be separated from viral enzymes by this procedure.

MATERIALS AND METHODS

Cells and viruses

3T3FL cells both uninfected (5) and infected with a cloned isolate of Moloney leukemia virus (3T3IC) (6) were provided by Drs. R. H. Bassin and D. K. Haapala, NCI.

Rauscher murine leukemia virus (MuLV) was obtained from Electro-Nucleonics Laboratories, Inc., Bethesda, Md., as a suspension of 10^{11} particles per ml. This preparation had been purified from tissue-culture fluids by two isopycnic bandings in sucrose density gradients.

The human candidate C-type virus from the cell line RD-114 (7, 8), maintained at NCI by Dr. Paul Peebles, was a gift of Dr. Peebles. Particulate material was concentrated 200 times from previously clarified ($10,000 \times g$) tissue culture fluids by centrifugation ($100,000 \times g$, 175 min) through 10% glycerol.

Abbreviations: MuLV, Rauscher strain of murine leukemia virus; S_2threitol, dithiothreitol.

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Chemicals and assays

Lyophilized bovine serum and poly(riboadenylic acid) were obtained from Miles Laboratories. Oligodeoxythymidylic acid (chain length 12–18 nucleotides) $[(dT)_{12-18}]$ was a product of P-L Biochemicals Co. Calf-thymus DNA and electrophoretically purified DNase were purchased from Worthington Biochemical Corp. Unlabeled deoxyribonucleotide triphosphates and dithiothreitol (S_2threitol) were products of Calbiochem. Cellulose powder (CF11) and phosphocellulose (P11, powder) were from Whatman, while dicyclohexylcarbodiimide and pyridine were obtained from Eastman Organic Chemicals. Radioactive thymidine triphosphate ([*methyl*-^{3}H]TTP, 12,000 cpm/pmol) was from New England Nuclear.

Polymerase assays

Fractions were assayed as described by Ross *et al.* (4) for RNA-dependent DNA polymerase and DNA-dependent DNA polymerase with the templates $poly(rA)\cdot(dT)_{12-18}$ and "nicked" calf-thymus DNA, respectively. All reaction mixtures (0.05 ml total volume) contained 80 mM Tris·HCl (pH 7.8), 0.1 mM S_2threitol, 20 μM [^{3}H]TTP, and 20 mM KCl. Reactions with $poly(rA)\cdot(dT)_{12-18}$ contained in addition 0.006 A_{260} units of polymer and 50 mM $Mn(OAc)_2$. Reactions with the "activated" DNA template (4) contained 0.2 A_{260} units of DNA, 6 mM $Mg(OAc)_2$, and 0.1 mM each of unlabeled dATP, dGTP, and dCTP. Assays were linear for 90 min at 37° at all enzyme concentrations tested and were saturated in template and substrate. Effluent profiles of enzyme activity from column chromatography were determined by assays of single aliquots from each fraction under the standard conditions described above. Values of total units are based on the linear regions of enzyme concentration curves. One unit of polymerase activity was defined as the amount of enzyme catalyzing the incorporation of 1 pmol of [^{3}H]TTP per hr at 37° into trichloroacetic acid-precipitable material.

Endonuclease assay

Endonuclease activity was measured by two methods. In the first method, crude and purified polymerase samples were incubated for 30 min at 37° in a 0.1 ml reaction mixture containing 73 μM λ DNA (a gift of Dr. John W. Little, NIAMD), 50 mM Tris·HCl (pH 7.8), 60 mM KCl, 6 mM $Mg(OAc)_2$, 0.1 mM S_2threitol, and 1.2 μg yeast RNA. The reaction was stopped by the addition of 0.9 ml 10% tri-

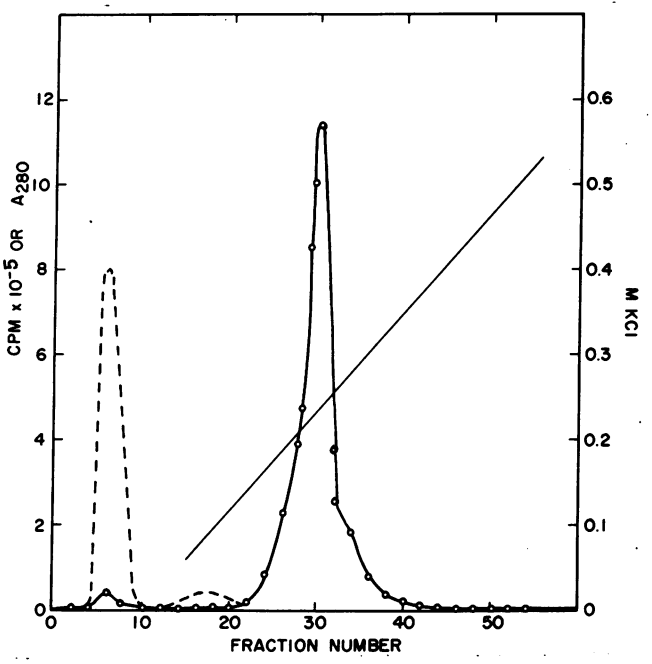

FIG. 1. Chromatography of MuLV DNA polymerase on $(dT)_{12-18}$-cellulose. 2 ml (2 mg protein) of dialyzed extract (S-100) of MuLV were applied to the column (0.9 × 12 cm) and eluted (see *Methods*). Aliquots (0.01 ml) of the 2.0-ml fractions were assayed in 0.05-ml reaction mixtures for 60 min at 37°. (- - -), A_{280}; (O), cpm [^{3}H]TTP incorporated with the poly(rA)·$(dT)_{12-18}$ assay; (——) M KCl.

chloroacetic acid and centrifuged at 4° for 10 min at 4,000 × *g*. The increase in acid-soluble absorbance at 260 nm was then determined.

In the second method, the conversion of closed covalent-circular (46 S) to linear (31 S) DNA was monitored. The DNA, enterotoxin plasmid DNA from *Escherichia coli*, labeled with [^{3}H]thymidine, was kindly supplied by Dr. Stanley Falkow, Georgetown University. Reaction mixtures containing equivalent amounts of DNA, 60 mM potassium phosphate (pH 7), 1 mM $MgCl_2$, and 10 units of either crude or purified RNA-dependent DNA polymerase or DNase I were incubated for 30 min at 37°, layered on separate 5–20% neutral sucrose gradients, and centrifuged at 41,000 rpm and 15° for 60 min in a SW41 rotor. 30 Fractions were collected from each gradient, and the movement of acid-precipitable counts from the position of intact to nicked circular DNA was determined.

Protein was determined by the method of Lowry *et al.* (9) with analytical control serum (bovine) as a standard. Salt concentrations were determined by conductivity readings on a Radiometer model CDM3 conductivity meter.

Preparation of $(dT)_{12-18}$-cellulose

$(dT)_{12-18}$ was coupled to cellulose by the method of Gilham (10). This chain length was chosen because of the reported preference of viral DNA polymerase for primers of this size (3, 4). A typical reaction mixture contained 5 g cellulose, 2 g dicyclohexylcarbodiimide, and 15 A_{260} units of $(dT)_{12-18}$ in 68 ml dry pyridine. Glass beads were included for stirring, and the flask was shaken for 5 days at room temperature. $(dT)_{12-18}$-cellulose was washed and packed into a column (0.9 × 12 cm) with 0.3 atm of N_2 pressure and $(dT)_{12-18}$ buffer (see column chromatography) until the absorbance of the effluent at 267 nm was within 0.01 of the buffer. The total absorbance of wash fractions indicated that 55%, or 8.3 units, of $(dT)_{12-18}$ were coupled to the cellulose. Columns were equilibrated with $(dT)_{12-18}$ buffer containing 0.5 mM $Mn(OAc)_2$ before use.

Solubilization of extracts for column chromatography

Cells were collected from tissue culture flasks by trypsinization and centrifugation. Packed cells were suspended in 1 ml of isotonic phosphate-buffered saline (pH 7.4) and sonicated for 3 min. The sonicate was then diluted with an equal volume of buffer containing 0.1 M Tris·HCl (pH 7.8)–1.0 M KCl, 2 mM S_2threitol–2% Triton X-100–40% (v/v) glycerol (disruption buffer). The cell suspension was then incubated for 30 min at 37° and centrifuged at 100,000 × *g* for 1 hr at 4°. The supernatant (S-100) was stored at −70°.

Virus samples were suspended in disruption buffer that had been diluted with an equal volume of water and treated as described for cell extracts.

Column chromatography

Affinity Chromatography. Samples were dialyzed for 1 hr against 250 volumes of buffer containing 10 mM potassium phosphate (pH 7.1)–1 mM S_2threitol–60 mM KCl–0.1% Triton X-100–20% (v/v) glycerol [$(dT)_{12-18}$ buffer] and, in addition, 0.5 mM $Mn(OAc)_2$. Samples were then applied to $(dT)_{12-18}$-cellulose columns, equilibrated with the same buffer, and eluted with a linear KCl gradient in $(dT)_{12-18}$ buffer containing no Mn^{+2}. Columns were washed with 1 M KCl and equilibrated with $(dT)_{12-18}$ buffer. Flow rates were maintained by pumping at 20 ml/hr. Columns were not used more than four times, since nucleases in the samples attacked oligonucleotides, with result antirregularity of elution profiles and smearing of polymerase activity throughout the column.

Ion-Exchange Chromatography. Phosphocellulose was washed extensively with 0.2 N NaOH, water, 0.1 N HCl in 50% ethanol, and—finally—water, until the pH was that of glass-distilled water. The pH was brought to 6.5 by addition of solid imidazole. The resin was then packed into a 0.9 × 15-cm column under 0.3 atm N_2 pressure. Columns were equilibrated, and samples were applied and eluted essentially as described by Ross *et al.*, except that all buffers contained 0.1% Triton X-100 (4).

Gel Filtration. A Sephadex G-100 column (1.5 × 90 cm) was equilibrated with 20 mM Tris·HCl (pH 7.8)–0.1% Triton X-100–0.1 mM KCl, 1 mM S_2threitol–0.1 mM EDTA–20% (v/v) glycerol. It was calibrated in separate experiments with blue dextran (molecular weight >2 × 10^6), bovine serum albumin (molecular weight 67,000), ovalbumin (molecular weight 45,000), chymotrypsinogen (molecular weight 25,000), and ribonuclease A (molecular weight 13,700). All of these markers were obtained from Pharmacia Fine Chemicals except for bovine serum albumin, which was from Pentex. Columns were pumped at a flow rate of 10 ml/hr, and 2.0-ml fractions were collected.

RESULTS

Purification of DNA polymerase from MuLV

Purified MuLV (1 ml, 10^{11} particles per ml) was disrupted in 1% Triton X-100–0.5 M KCl as described in *Methods*. The

solubilized fraction containing polymerase activity was dialyzed, applied to a $(dT)_{12-18}$–cellulose column, and eluted with a KCl gradient as shown in Fig. 1. Although Mn^{+2} was used for application of sample in the experiments reported here, its omission does not alter the binding of the polymerase to the column or its position of elution. After the initial appearance of enzyme activity in the wash peak, as many as 20 column volumes of wash buffer eluted no additional polymerase activity. In the experiment of Fig. 1, a salt gradient of 0.06–0.8 M KCl was applied after the front peak emerged. The maximum DNA polymerase activity is eluted at 0.21 ± 0.01 M KCl.

The amount of polymerase activity found in the front wash peak varies and is apparently an overloading phenomenon. This activity binds to $(dT)_{12-18}$–cellulose if applied again and chromatographs like viral polymerase on phosphocellulose. An additional protein peak is obtained with a 1 M KCl wash. This peak varies in size and generally contains no DNA polymerase activity for virus samples.

When peak enzyme fractions eluted by 0.2 M KCl are assayed for DNA polymerase activities primed by poly(rA)·$(dT)_{12-18}$ and "nicked" DNA, a constant ratio of about 30:1 poly(rA)·$(dT)_{12-18}$:DNA is observed. Enzyme activity was stable for at least 6 weeks if stored at −70° without repeated freezing and thawing.

Table 1 follows the purification of protein and increase in specific activity as far as possible. The large increase in total units on $(dT)_{12-18}$ chromatography has been a consistent finding. An increase in units was also noted by Hurwitz and Leis (11) after ammonium sulfate precipitation of a crude MuLV extract. The presence of Triton X-100 in the buffers was essential for stability of polymerase activity but made protein determinations unreliable below concentrations of 0.08 mg/ml. Protein could not be followed as A_{280} below values of 0.1 because of the high ultraviolet absorbance of the detergent.

Enzyme purified by $(dT)_{12-18}$–cellulose chromatography contained no detectable endonuclease activity. This activity was monitored against both λ DNA and tritiated circular plasmid DNA. Samples of either crude (S-100) or purified (dT_{12-18}–cellulose) fractions containing 10–20 units of RNA-dependent DNA polymerase activity were incubated with

TABLE 1. *Purification of polymerase activity from MuLV*

Fraction	Total protein* mg	Total units	Specific activity units/mg†
Virus	4.4	50,000	11,400
S-100	2.0	48,500	24,300
Front wash (Fractions 4–9)	1.1	1,750	1,590
Front fractions (10–22)	0.4	820	2,050
1 M KCl wash	0.4	0	0
Enzyme peak (Fractions 23–52)	<0.08	84,700	>1,060,000

Polymerase assays, regardless of template, contained in 0.05 ml: 80 mM Tris·HCl (pH 7.8), 0.1 mM S₂threitol, 20 μM [³H]-TTP, 20 mM KCl, 0.006 A_{260} units of poly(rA)·$(dT)_{12-18}$, and 0.5 mM $Mn(OAc)_2$.

* Protein was determined by the method of Lowry (9).

† One unit is defined as that amount of enzyme that converts 1 pmol of TTP per hr at 37° to acid-precipitable material.

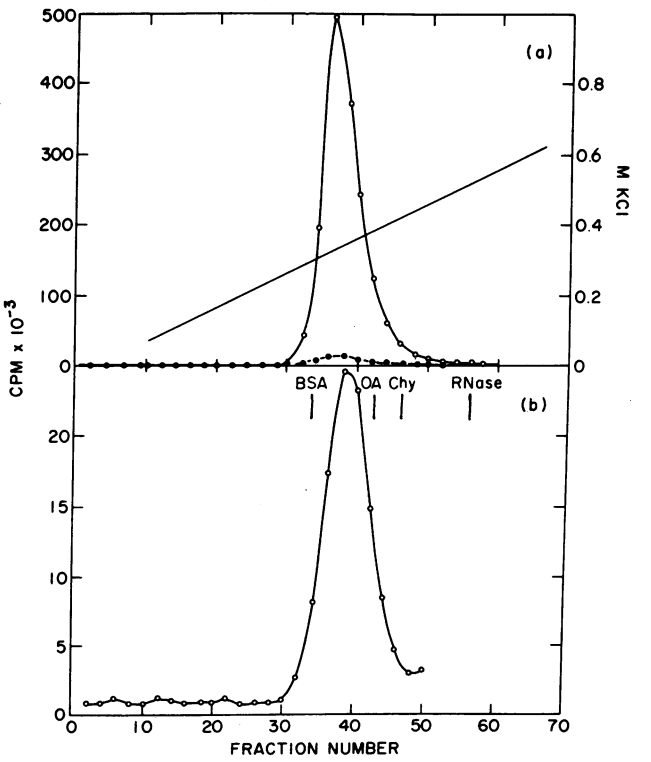

FIG. 2. (*a*) Elution of MuLV DNA polymerase purified by $(dT)_{12-18}$ from phosphocellulose. 1 ml of pooled fractions from the $(dT)_{12-18}$ column was applied to a column (0.9 × 15 cm) of phosphocellulose and eluted (see *Methods*). (○) RNA-dependent DNA polymerase activity; (●) DNA-dependent DNA polymerase activity. (*b*) Elution of MuLV DNA polymerase from Sephadex G-100. A 1-ml sample of a pool of fractions 35–37 from phosphocellulose column of Fig. 2(*a*) was applied to a previously calibrated column (1.5 × 90 cm) of Sephadex G-100 and eluted (see *Methods*). *Lines* indicate elution positions of bovine serum albumin (BSA), ovalbumin (OA), chymotrypsinogen (Chy), and ribonuclease A(RNase). Polymerase assays of the 2.0-ml fractions of both columns were performed on 0.01-ml aliquots as in Fig. 1.

these substrates and analyzed as described in *Methods*. Endonuclease activity was detectable by both techniques in the crude samples and was absent in the purified enzyme.

To further test the catalytic purity of the eluted enzyme, we examined the elution pattern of RNA- and DNA-dependent DNA polymerase activities from phosphocellulose and calibrated Sephadex G-100 columns. The elution profiles for the columns are shown in Fig. 2. In both cases the RNA- and DNA-dependent DNA polymerase activities cochromatograph, showing a ratio of RNA to DNA dependence of about 30:1. The polymerase activity eluted from phosphocellulose at about 0.3 M KCl. This elution position is characteristic in this laboratory for phosphocellulose chromatography of DNA polymerases from feline (Rickard), murine (Gross, Rauscher), and avian (RAV-1) viruses.

The elution volume of the viral polymerase from a calibrated Sephadex G-100 column corresponded to a molecular weight of 50,000, assuming that the protein is globular. This value is significantly lower than that of 70,000 previously obtained for this protein (4). MuLV polymerase purified by phosphocellulose alone eluted in the same position on the same Sephadex column. Cuatrecasas has recently confirmed

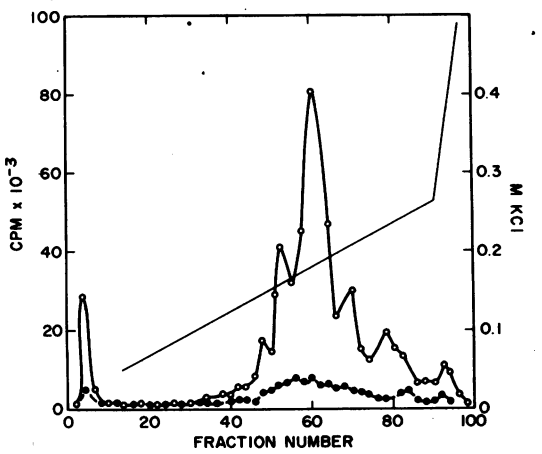

FIG. 3. Chromatography of RNA-dependent DNA polymerase activity from the virus of RD-114 cells. 1 ml of a dialyzed extract of RD-114 virus was applied to the $(dT)_{12-18}$-cellulose column (0.9 × 12 cm), eluted, and assayed for RNA (○)- and DNA (●)-dependent DNA polymerase activity as described for MuLV in Fig. 1, except that the salt gradient ran from 0.06–0.26 M KCl, followed by a 1 M KCl salt wash.

the applicability of gel filtration in buffer containing from 0.05 to 0.5% Triton X-100 (12).

Purification of DNA polymerase from RD-114 virus

A virus pellet from tissue culture fluids from the RD-114 cell line (7, 8) was solubilized as described for MuLV, and a 100,000 × *g* supernatant was obtained. This solution was dialyzed and applied to the $(dT)_{12-18}$-cellulose column as for MuLV. Elution of DNA polymerase activity is shown in Fig. 3. The enzyme binds firmly to the column and elutes in the same region of ionic strength as the polymerase from MuLV. The ratio of poly(rA)·$(dT)_{12-18}$ to "nicked" DNA-primed DNA polymerase activities of the eluted enzyme is about 8.4:1, showing a preference for direction by poly(rA)·$(dT)_{12-18}$. The irregularity of the elution profile in this experiment may be due to the fact that this column had already been used four times.

Specificity of $(dT)_{12-18}$-cellulose for "viral"-type enzyme

Extracts from uninfected 3T3FL cells and from the same cells infected with the IC isolate of Moloney leukemia virus (6) were applied to columns of $(dT)_{12-18}$-cellulose and eluted with a salt gradient. Fractions were assayed for both RNA- and DNA-directed DNA polymerase activity (Fig. 4). The extract from infected cells (Fig. 4*a*) clearly contains an RNA-directed DNA polymerase activity that binds to the column and elutes at 0.2 M KCl, as did the polymerase from MuLV.

The uninfected cell extract (Fig. 4*b*) shows no polymerase activity eluted by the salt gradient and a small peak of DNA-directed DNA polymerase activity that elutes in the final 1 M KCl wash. Thus, the normal cellular polymerases of this mammalian cell line do not bind to the $(dT)_{12-18}$-cellulose column under these conditions. In order to examine this point further, the wash peak from uninfected 3T3FL cells was applied to a phosphocellulose column, and the elution pattern of polymerase activity from this column was identical to that obtained in this laboratory for an uninfected cell extract applied directly.

Polymerase activity from cultures containing mycoplasma does not bind to $(dT)_{12-18}$-cellulose.

DISCUSSION

The $(dT)_{12-18}$-cellulose column is a simple and efficient means of purifying viral RNA-dependent DNA polymerases. Neither the binding of polymerase to this column nor the ionic strength of its elution is influenced by the omission of Mn^{+2}, while the catalytic activity of this enzyme from mammalian viruses is greatly enhanced by this cation (13). These observations suggest either that the enzyme under noncatalytic conditions contains sufficient bound cation for binding to $(dT)_{12-18}$ or that the role of Mn^{+2} in catalysis is not primarily the binding of enzyme to primer.

Recent reports of the presence of poly(rA) sequences in viral RNA (14, 15) raise the possibility that polymerase is bound to a small amount of poly(rA), which in turn binds to oligo(dT). The presence of such a complex after the high salt treatment involved in the solubilization process would suggest a specific molecular interaction in which the RNA sequence, would be an integral part of the functional enzyme. Thus,

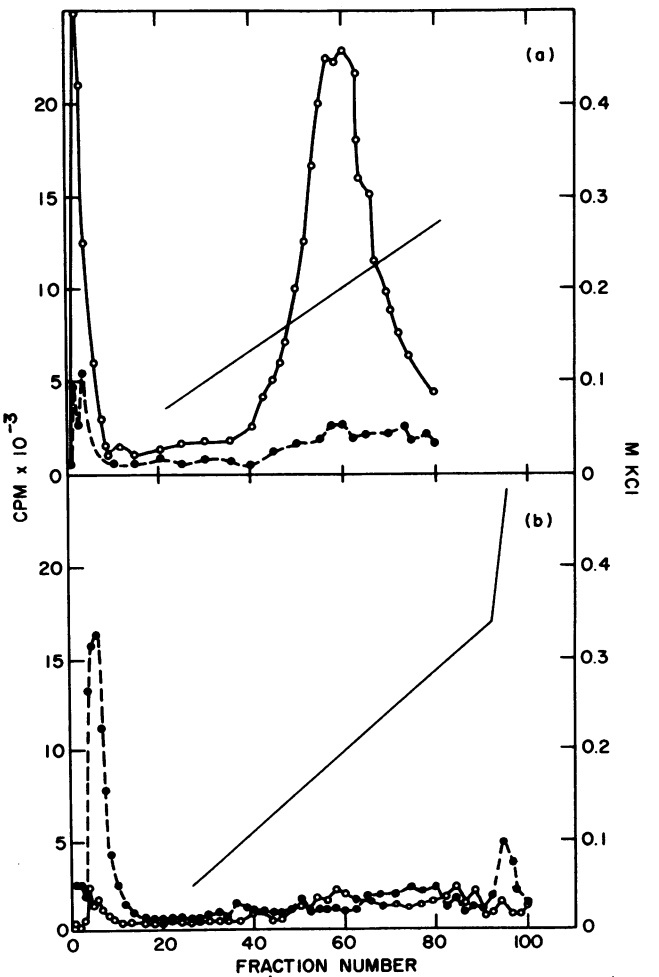

FIG. 4. Chromatography of extracts from 3T3IC (*a*) and 3T3FL (*b*) cells on a $(dT)_{12-18}$-cellulose column (0.9 × 12 cm). (*a*) 1 ml (5 mg protein) of a crude extract was dialyzed, applied, eluted, and assayed for RNA (○)- and DNA (●)-dependent DNA polymerase activity as described for Fig. 3. (*b*) Same as (*a*) except that 2 ml (19 mg of protein) of a crude extract was applied.

specific binding of viral DNA polymerase to the $(dT)_{12-18}$–cellulose column may be due either to a binding site for $(dT)_{12-18}$ on the protein molecule or to the existence of an RNA–enzyme molecular complex in which poly(rA) binds to $(dT)_{12-18}$.

In addition to the usefulness of $(dT)_{12-18}$–cellulose columns for purification of known viral polymerases, this procedure can discriminate between viral and cellular polymerase activities. Other workers have shown that viral polymerase has greater catalytic activity than cellular polymerases with poly(rA)·$(dT)_{12-18}$ templates (3, 4). These differences may be reflected in differences in binding constants sufficient to account for the failure of cellular polymerases to bind to the $(dT)_{12-18}$–cellulose column.

The enzyme isolated from the RD-114 virus, unlike polymerases from feline, rat, or hamster viruses, is not inhibited by antisera prepared against the polymerase of Rauscher MuLV (16). The binding of this enzyme by the $(dT)_{12-18}$–cellulose column supports the interpretation that viral enzymes bind to the resin because of their affinity for the $(dT)_{12-18}$ primer and not because of similarities unrelated to mechanism of action. In addition this experiment shows that while binding to this column will not be useful for characterizing the species of origin of a new viral polymerase, it may provide an additional criterion of whether human material does contain a viral polymerase.

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