Proc. Nat. Acad. Sci. USA
Vol. 69, No. 2, pp. 393–397, February 1972

Affinity Chromatography of RNA-Dependent DNA Polymerase from RNA Tumor Viruses on a Solid Phase Immunoadsorbent

(murine leukemia virus/feline sarcoma-leukemia virus complex/Rous sarcoma virus)

DAVID M. LIVINGSTON, EDWARD M. SCOLNICK, WADE P. PARKS, AND GEORGE J. TODARO

Viral Leukemia and Lymphoma Branch, Viral Carcinogenesis Branch, National Cancer Institute, Bethesda, Maryland 20014

Communicated by Robert J. Huebner, November 11, 1971

ABSTRACT **A solid phase immunoadsorbent specific for RNA-dependent DNA polymerase from murine and feline RNA tumor viruses has been prepared. The enzymes from murine and feline virus but not from avian virus bind to columns of this material. Bound enzymes can be eluted in active form. This method has permitted selective purification of viral enzyme from crude extracts of virus-transformed cells, since the immunoadsorbent has no affinity for cellular DNA polymerases.**

Solid-phase affinity chromatography of proteins is based on the principle that an insoluble compound or macromolecule with selective affinity for a given protein will specifically bind to that protein (1). The method has proven valuable for the rapid and selective purification of enzymes from partially purified mixtures and crude cell extracts (2). One application of this method involves the specific binding of an enzyme to antibody that is covalently coupled to Sepharose (3). We report here the development and properties of such a selective immunoadsorbent for RNA-dependent DNA polymerase from murine leukemia virus (MuLV). Columns of this material bind the RNA-dependent DNA polymerase of RNA tumor viruses of mouse and feline origin, and the bound enzymes can be eluted in active form.

MATERIALS AND METHODS

Viruses. The Rauscher strain of murine leukemia virus (R-MuLV), the Gardner strain of the feline sarcoma–leukemia virus complex (G-FeSV), and the Schmidt–Ruppin strain of Rous sarcoma virus (SR-RSV), were obtained from Electro-Nucleonics, Inc., Bethesda, Md. They were purified by sucrose density gradient centrifugation before use.

Cells. BALB/3T3 cells producing no detectable virus and MSV-24 clone 39, a line of transformed BALB/3T3 cells producing both sarcoma virus and Moloney leukemia virus (4), were grown as described (5).

Chemicals. Cyanogen bromide was obtained from Eastman Organic Chemicals, Rochester, N.Y.; Sepharose 4B from Pharmacia, Piscataway, N.J.; and [methyl-^{3}H]TTP (specific activity 10 Ci/mmol) from Schwarz–Mann Research Lab, Orangeburg, N.Y. Polyriboadenylic acid (poly rA) was obtained from P-L Biochemical Co., Milwaukee, Wis.; and oligodeoxythymidylic acid (oligo dT) (chain length = 12–18 nucleotides) from Collaborative Research Co., Waltham, Mass. Polydeoxythymidylic acid (poly dT) was obtained from Miles Laboratories, Kankakee, Ill.

Isolation and Purification of Rabbit Antipolymerase and Control IgG. The methods used for rabbit immunization, serum isolation, and IgG purification have already been described (6, 7). The only modification was that the DEAE-cellulose chromatography of the dialyzed 50% ammonium sulfate serum fraction was performed in 0.01 M potassium phosphate, pH 6.8, rather than at pH 8.0.

Preparation of Antipolymerase and Control IgG-Coupled Immunoadsorbents. Procedures for the washing, activation of, and the ensuing IgG coupling to Sepharose 4B were those described by Wilchek *et al.* (8). The coupling reaction for antibody and control IgG was performed in 0.10 M $NaHCO_3$ (pH 8.0) for 16 hr, at 4°C, at a protein concentration of 3 mg/ml and a Sepharose/protein ratio of 30:1 (w/w). Under these conditions, over 90% of the added protein was coupled, and the IgG content of both Sepharose conjugates was estimated to be 33 mg/g. Equivalent volumes (about 2–3 ml) of each of the coupled, washed Sepharose–protein conjugates were then poured into plastic syringes (1.3 × 3 cm) with polyethylene outflow tubes. Buffers and solutions used during the chromatography procedure included: Buffer A [0.05 M Tris·HCl (pH 7.8)–0.30 M KCl–2% (v/v) Triton X-100–1% (w/v) bovine serum albumin (BSA; Armour)]; Buffer B [0.05 M Tris·HCl (pH 7.8)–0.30 M KCl]; Solution C [0.20 M NH_4OH (1.35 ml concentrated NH_4OH per 100 ml final volume)–0.30 M KCl–1% BSA]; Solution D [1.0 M NH_4OH (6.75 ml of concentrated NH_4OH per 100 ml final volume)–0.30 M KCl–1% BSA]. Columns were washed with 20 column volumes of Buffer B and washed subsequently with 4 column volumes of Buffer A before initial use.

Preparation of Crude Cellular Extracts. Crude extracts of BALB/3T3 and MSV-24C139 cells were prepared as follows: Cells were grown in 32-oz. prescription bottles in Dulbecco's modified Eagle's medium with 10% (v/v) calf serum (Colorado Serum Co.). The cells were scraped into 0.05 M phosphate-buffered saline (PBS) (pH 7.4), washed four times with PBS, suspended in 0.05 M Tris·HCl (pH 7.8)–1 mM EDTA (Na_2)–14mM 2-mercaptoethanol–2.0 M potassium chloride, and sonicated for 1 min at 4°C. 3–4 Volumes of the above buffer were used per volume of packed cells. The solu-

Abbreviations: R-MuLV, Rauscher strain of murine leukemia virus; G-FeSV, Gardner strain of feline sarcoma–leukemia virus complex; SR-RSV, Schmidt–Ruppin strain of Rous sarcoma virus; MSV, mouse sarcoma virus; BSA, bovine serum albumin.

tion was then made 1% (v/v) in Triton X-100, stirred at 4°C for 30 min, and then centrifuged at 100,000 × *g* for 60 min. The supernatant (S-100) was stored at −170°C before use.

Preparation of Partially Purified RNA-Dependent DNA Polymerase from MuLV, FeSV, and SR-RSV. The Sephadex G-100 fractions with maximal polymerase activity were obtained from virions disrupted with Triton X-100 by the method of Ross *et al.* (5), pooled, and frozen at −170°C before further use.

Polymerase Assays. Assays were performed as described (5); the final concentration of KCl in the reaction mixtures was 0.07 M. Viral polymerase was assayed with poly rA · oligo $dT_{(12-18)}$ as template, since only low polymerase activity has been observed in uninfected cells when this template was used (5). One unit of activity is defined as an amount of enzyme that catalyzes the polymerization of 0.02 pmol of [^{3}H]TTP in 10 min at 37°C.

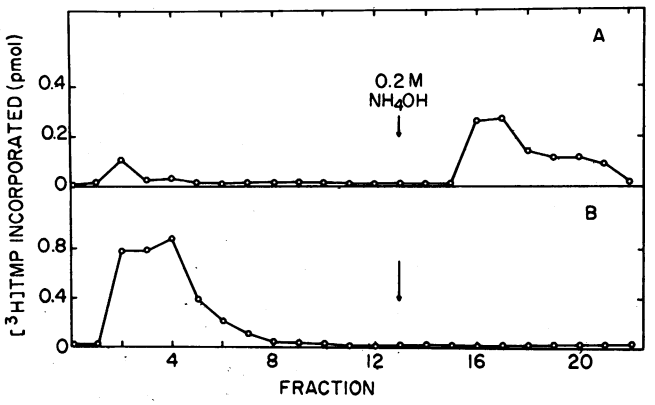

FIG. 1. Chromatography of RNA-dependent DNA polymerase from R-MuLV on the antipolymerase and control IgG Sepharose columns. 72 μg of Sephadex G-100 purified enzyme were applied to the antipolymerase IgG column (*A*) and the control IgG column (*B*). The fraction volume was 2 ml. The first eight fractions represented the Buffer A wash, the next four the Buffer B wash, and the remainder, elution with Solution C. Operation of the columns and assay conditions are described in *Methods*. 0.02-ml aliquots of each fraction were assayed in 0.20-ml reaction mixtures for 60 min at 37°C.

Operation of Antipolymerase and Control IgG Columns. All operations were performed at 4°C. Samples of partially purified viral RNA-dependent DNA polymerase or cell extracts (S-100) were made 2% in Triton X-100 and 1% in BSA and then applied to the column. After the sample had fully entered the Sepharose, 0.10 column volume of Buffer A was applied to the column; after it had also entered, the column outflow tube was clamped to halt flow from the column for 5–10 min. The elution was resumed with Buffer A, with the column flow rate maintained at 0.4–0.5 ml/min. At an elution volume indicated in each experiment, washing with Buffer B was begun and the flow rate was adjusted to about 0.6–0.8 ml/min. At the completion of this wash, Solution C was applied and elution with it was continued for 8–10 column volumes, i.e., until there was no further elution of active enzyme. 2- to 3-ml fractions were collected.

Aliquots of each fraction were assayed at 37°C for 60 min. After each chromatographic experiment, the columns were washed with three column volumes of Solution D and then re-equilibrated in Buffer B. The columns were subsequently equilibrated in Buffer A for use in the next experiment. Little or no loss in binding capacity of the antibody column was noted over a 2-month period despite repeated use.

Protein determinations were performed by the method of Lowry *et al.* (9); BSA was used as a standard. Appropriate controls for buffer and potential interfering nonprotein substances were routinely performed.

Acrylamide Gel Electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gels were prepared as described by Shapiro *et al.* (10). 6-cm long gels, containing 10% acrylamide–0.3% bis-acrylamide–0.1% SDS–0.1 M sodium phosphate (pH 7.2) were run at 5 mA/gel. The electrophoresis buffer was 0.1 M sodium phosphate (pH 7.2)–0.1% (v/v) SDS. Samples were heated at 65°C for 10 min in buffer containing 0.1% SDS, 0.01 M sodium phosphate (pH 7.2), 0.14 M 2-mercaptoethanol, 10% (v/v) glycerol, and 0.02% bromophenol blue as a tracking dye before application to the column. Electrophoresis was discontinued when the tracking dye had reached the bottom of the gel; the gels were then fixed and stained in 0.25% (w/v) Coomassie brilliant blue in methanol–acetic acid–water (5:1:5). After 18 hr, gels were destained in 7.5% acetic acid–5% methanol.

RESULTS

Binding and elution of RNA-dependent DNA polymerase from R-MuLV

Identical aliquots of partially purified enzyme from R-MuLV were applied to the antipolymerase and control IgG columns (Fig. 1*A* and *B*). All of the activity appeared in the wash-through fractions of the control column. Less than 5% of the applied activity appeared in the wash-through fractions from the antibody column, while application of Solution C resulted in elution of appreciable RNA-dependent DNA polymerase activity. It is important to remove all traces of Triton X-100 from the column wash solvent before elution with Solution C, since addition of the detergent to the NH_4OH solutions led to significant inactivation of the enzyme.

The overall yield of the enzyme eluted with Solution C from the antipolymerase column was 30% relative to that appearing in the wash-through fractions from the control column. The yields in repeated experiments have varied from 25–40%.

Binding and elution of RNA-dependent DNA polymerase from FeSV and S-R RSV

Partially purified RNA-dependent DNA polymerase from feline virus was also applied to the antipolymerase and control columns as shown in Fig. 2*A* and *B*, respectively. Again, all of the activity eluted in the wash-through fractions of the control column, and none was eluted with Solution C. Approximately one-third of the activity appeared in the fraction that washed through the antibody column, and application of Solution C eluted the remaining activity. In this instance, the column capacity was exceeded since application of less material led to virtually complete binding. Furthermore, reapplication of feline viral enzyme from the antipolymerase column wash-through fractions bound to and was eluted from the antipolymerase column in a subsequent experiment.

The total yield of activity from the antibody column with the feline virus enzyme has consistently approached 100% of comparable material washing through the control IgG column.

The persistence of feline viral polymerase activity throughout the Buffer B wash suggests that the affinity of the immunoadsorbent for the feline polymerase is lower than that for the murine enzyme. Comparable amounts of polymerase activity from the Schmidt–Ruppin strain of Rous sarcoma virus wash through the anti-MuLV polymerase (Fig. 2*C*) and control IgG columns (Fig. 2*D*), indicating that this enzyme does not bind appreciably to the immunoadsorbent under these conditions.

These results demonstrate that the RNA-dependent DNA polymerase of the feline leukemia–sarcoma virus is immunologically related to the murine leukemia viral polymerase, since it binds to the antimurine polymerase immunoadsorbent; no such relationship is apparent for the avian sarcoma virus polymerase.

Purification of RNA-dependent DNA polymerase of MSV (MuLV) from crude extracts of virus-producing cells

Aliquots of supernatant S-100 (see *Methods*) from the murine sarcoma virus-transformed BALB/3T3 line were applied to both the control and antipolymerase columns. All of the polymerase activity appeared in the wash fractions from the control IgG column (Fig. 3*B*), while only 24% of the activity was in fractions from the antipolymerase column (Fig. 3*A*). Thus, most of the applied viral enzyme did bind to the antibody column. Application of Solution C to the column again resulted in the release of active polymerase (yield = 25% of that in the control column wash-through fractions) from the antipolymerase but not the control IgG column.

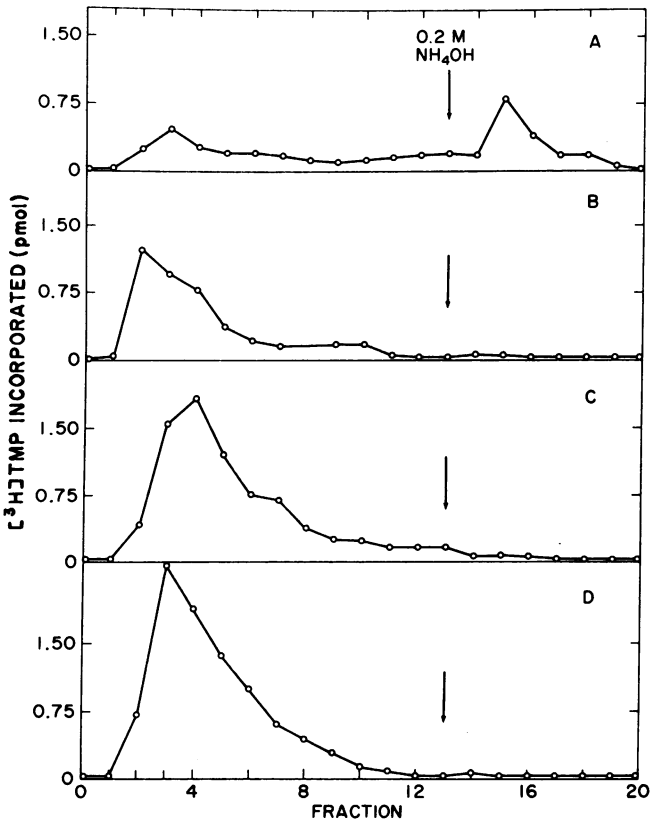

FIG. 2. Chromatography of RNA-dependent DNA polymerase from G-FeSV and SR-RSV on the antipolymerase and control IgG columns. 24 μg of RNA-dependent DNA polymerase from G-FeSV that was purified on Sephadex G-100 were applied to the antipolymerase IgG (*A*) and control IgG column (*B*). 24 μg of SR-RSV enzyme that was purified on Sephadex G-100 were applied to the antipolymerase IgG column (*C*) and the control IgG column (*D*). Fraction volume, distribution, and column operation were exactly as described in the legend to Fig. 1 and *Methods*. 0.02-ml aliquots of each fraction were assayed as described in the legend to Fig. 1.

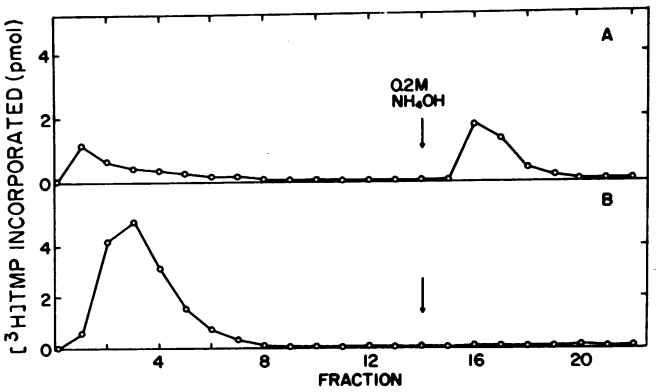

FIG. 3. Chromatography of a crude extract of MSV-24-C139 cells on the antipolymerase and control IgG column. 3.8 mg of cell extract (S-100) (see *Methods*) were applied to the antipolymerase IgG column (*A*) and the control IgG column (*B*). 3-ml fractions were collected and assayed as described in *Methods*. Fractions 1–11 represent the Buffer A wash, 12–14 the Buffer B wash, and 15–22 the Solution C eluate. 0.02-ml aliquots of each fraction were assayed as described in the legend to Fig. 1.

Absence of binding and elution of host polymerase activity from crude extracts of normal cells

Extracts of BALB/3T3 cells were applied to both columns. The amounts of protein chromatographed were identical to those applied to the columns in the previous experiment with the MSV-24C139 cell extract. As shown in Fig. 4, practically no activity was detected when poly rA·oligo $dT_{(12-18)}$ was used as a template. In some experiments with the antibody column, small amounts (less than 0.02 pmol) of activity have

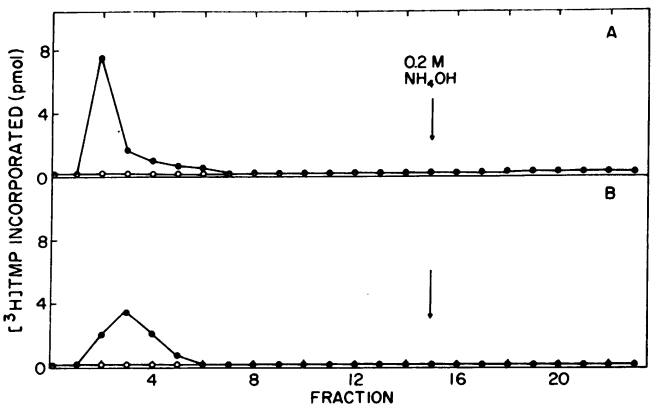

FIG. 4. Chromatography of a crude extract of BALB/3T3 cells on the antipolymerase and control IgG columns. 3.8 mg of S-100 were applied to the antipolymerase IgG (*A*) and the control IgG column (*B*). All operations were identical to those noted in the legend to Fig. 3. Each fraction was assayed separately with 0.02 A_{260} of poly rA·oligo $dT_{(12-18)}$ (O—O) and 0.02 A_{260} of poly rA·poly dT (●—●). 0.02-ml aliquots were assayed as described in the legend of Fig. 1.

been detected with this template in the fractions eluted with Solution C; the significance of these observations is presently under investigation. We therefore chose to measure uninfected cell polymerase activity with poly rA · poly dT as a template, since this heteropolymer is useful for detection of host cell DNA polymerase activity as well as viral RNA-dependent DNA polymerase (5). Under these conditions, by use of poly rA · poly dT or activated DNA (data not shown) as template,

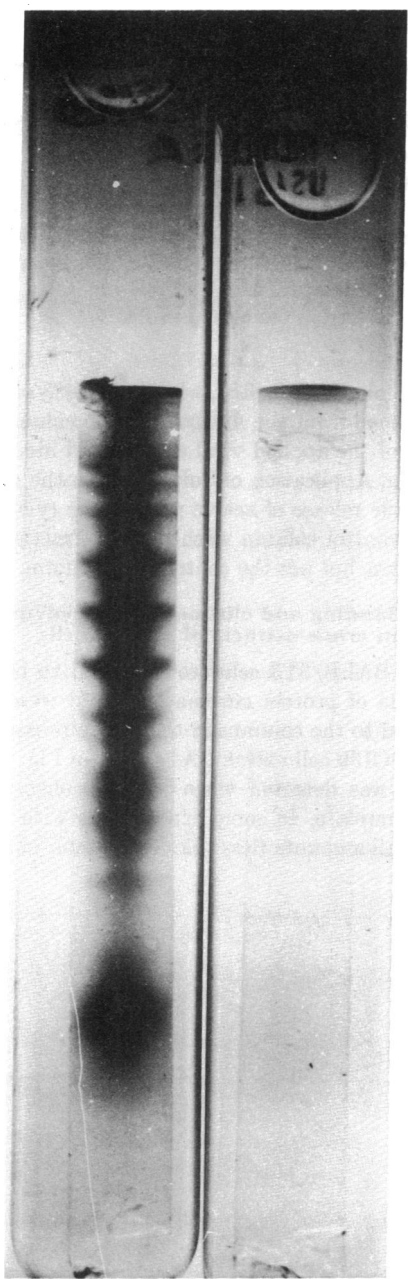

FIG. 5. SDS-polyacrylamide gel electrophoresis of crude MSV-24-C139 extract and antipolymerase IgG column-purified enzyme from these cells. 6.3 μg of crude extract protein (127 units of RNA-dependent DNA polymerase activity) (*left gel*) and 250 units of RNA-dependent DNA polymerase from the peak fraction of the material eluted with Solution C from an experiment identical to that described in the legend to Fig. 3*A*, but performed in the absence of BSA in Solution C (*right gel*) were electrophoresed as described in *Methods*.

DNA polymerase activity is found in the wash but not in the fractions of both columns eluted with Solution C.

Degree of purification of viral RNA-dependent DNA polymerase from crude cellular extracts

Protein could not be detected in the 0.2 M NH_4OH fractions from the anti-polymerase column after application of up to 3.8 mg of protein from the crude extract of MSV-24-C139 cells. Therefore, a calculation of specific activity of the purified enzyme from cells is not possible. However, by an examination of SDS-disc gel electrophoretograms of the starting material and the peak fraction of the material eluted with NH_4OH, a pictorial indication that the specific activity of the viral enzyme has been significantly increased can be obtained (Fig. 5). On the left is a gel to which was applied 6.3 μg of MSV-24-C139 protein (127 units of activity). On the right is a comparable gel to which was applied 250 units of enzyme activity from the peak fraction of the material eluted with NH_4OH. The enzyme applied to the latter gel had been eluted from the antipolymerase column with Solution C from which albumin was omitted. There is a marked decrease in the number and intensity of the bands in the purified relative to the unfractionated material. Indeed only two faint bands were detectable, compared to the more than 10 bands in the crude material.

DISCUSSION

The antibody used in these experiments was prepared by immunization of rabbits with partially purified RNA-dependent DNA polymerase from R-MuLV (6). From studies performed with soluble antibodies, it can be shown that this immunoglobulin preparation inhibits viral RNA-dependent DNA polymerase from the known C-type viruses of murine and feline origin, but not the polymerase of avian viruses (7). Predictably, the solid phase immunoadsorbent prepared with this immunoglobulin binds the enzymes from murine and feline leukemia virus but not from the Schmidt–Ruppin strain of Rous sarcoma virus. Enzyme can then be eluted from the Sepharose–antibody conjugate at alkaline pH (10.6) in 0.20 M NH_4OH–0.30 M KCl–1% BSA. Presumably, at this pH and under these conditions, the structure of the immunoglobulin is so altered that its affinity for the enzyme is greatly reduced. NH_4OH has been used previously for the elution of an azo uridinediphosphate peptide from Sepharose–RNase and of haptens from antihapten-antibody Sepharose columns (11, 8).

Binding of the enzyme is to a specific immunoglobulin, since a control absorbent prepared with equivalent amounts of purified IgG from an unimmunized animal revealed no affinity for the RNA-dependent DNA polymerase under these conditions. To observe specific binding, however, 0.30 M KCl, 1% BSA, and 2% Triton X-100 must be present in the application and wash-through buffers, since in their absence, there is substantial nonspecific binding of the enzyme to both unsubstituted Sepharose and the control IgG column. Under the conditions used, however, only specific binding occurs, since enzyme is not retained by unsubstituted Sepharose or the control column.

The yield of DNA polymerase after elution of the antibody column with NH_4OH is 25–40% relative to the amount of activity in the wash-through fractions from the control column. The addition of 0.10 volumes of solution C to a standard reaction mixture is not inhibitory to Sephadex G-

100-purified viral enzyme. Thus, it is unlikely that the enzyme eluted from the antipolymerase column is being incompletely measured due to the addition of inhibitory substances from solution C to the assay mixture. Attempts to elute more enzyme activity with higher concentrations of NH_4OH have thus far been unsuccessful. The observed losses in activity may be related to an alteration in enzymatic properties caused by prolonged exposure of the enzyme to an alkaline medium. It is also possible that the loss of activity is, in part, a result of irreversible changes in enzyme structure that accompany antibody binding. Interestingly, the feline enzyme is eluted in much higher yield than the murine enzyme, suggesting structural differences between these two proteins.

The antibody affinity column also binds viral polymerase from a crude extract of MSV-transformed cells. This enzyme can also be eluted from the column in active form. In order to determine whether any cellular DNA polymerases might also bind and be eluted, we applied an identical quantity of protein from a crude extract of the untransformed BALB/3T3 cells (Fig. 4). Host cell DNA polymerase is not retained on the column, consistent with the observation that the anti-MuLV polymerase antibody does not inhibit either of the known host cell DNA polymerases (5). Thus, the column can be used to purify selectively the viral polymerase from cellular polymerases. Since the enzyme eluted with Solution C is free of host-cell DNA polymerase, it should be possible to assay it unambiguously with synthetic templates that allow a more sensitive measurement of viral RNA-dependant DNA polymerase activity.

Since the procedure involves only two steps, the preparation of a crude extract and the affinity column procedure, it is rapid and simple. Thus, it should allow identification of viral enzyme from crude mixtures where activity might not be readily detected or distinguished in the impure state or even after conventional separation methods. It should be possible to obtain small quantities of enzyme in concentrated form from large volumes of extracts or tissue culture medium. These properties make affinity chromatography of the viral RNA-dependent DNA polymerase useful for the recognition of other members of this interesting class of enzymes. It should, however, be noted that suitable conditions for elution of viral enzymes of other species from their specific immunoadsorbents might be quite different from those reported here.

ACKNOWLEDGMENT

We thank Lois Serxner and Leslie Killeen for expert technical assistance. This work was supported in part by a contract from the Special Virus Cancer Program, National Cancer Institute, National Institutes of Health.

REFERENCES

1. Cuatrecasas, P. & Anfinsen, C. B. (1971) *Annu. Rev. Biochem.* **40**, 259–278.
2. Cuatrecasas, P., Wilchek, M. & Anfinsen, C. B. (1968) *Proc. Nat. Acad. Sci. USA* **61**, 636–643.
3. Melchers, F. & Messer, W. (1970) *Eur. J. Biochem.* **17**, 267–272.
4. Todaro, G. J. & Aaronson, S. A. (1969) *Virology* **38**, 174–179.
5. Ross, J., Scolnick, E. M., Todaro, G. J. & Aaronson, S. A. (1971) *Nature* **231**, 163–167.
6. Aaronson, S. A., Parks, W. P., Scolnick, E. M. & Todaro, G. J. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 920–924.
7. Parks, W. P., Scolnick, E. M., Ross, J., Todaro, G. J. & Aaronson, S. A., *J. Virol.* in press.
8. Wilchek, M., Bocchini, V., Becker, M. & Givol, D. (1971) *Biochemistry* **10**, 2828–2834.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J., (1951) *J. Biol. Chem.* **193**, 265–275.
10. Shapiro, A. L., Vinuela, E., & Maizel, J. V., Jr. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815–820.
11. Givol, D., Weinstein, Y., Gorecki, M. & Wilchek, M. (1970) *Biochem. Biophys. Res. Commun.* **38**, 825–830.