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Purification of Avian Myeloblastosis Virus DNA Polymerase by Affinity Chromatography on Polycytidylate-Agarose

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Polycytidylic acid [poly(rC)] covalently linked to cyanogen bromide-activated agarose is an effective affinity matrix for the RNA-dependent DNA polymerase from avian myeloblastosis virus. Poly(rC)-agarose is capable of binding large quantities of avian myeloblastosis DNA polymerase, which is then eluted by using a linear KCl gradient of increasing concentration. The DNA polymerase isolated from crude, detergent-disrupted virions by a single pass through columns of poly(rC)-agarose appears nearly homogeneous (approximately 90% pure) as determined by sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis. Complete recovery of input enzymatic activity was obtained. Results suggest that polyribonucleotide columns may provide a high-yield, rapid method for the purification of oncornaviral DNA polymerase.

Affinity chromatography has been widely used as a technique for the purification of biological macromolecules by utilizing the specific reversible interactions occurring between such macromolecules and their insolubilized effectors (3-5). The application of this technique to the isolation of DNA polymerases from oncornaviruses was first reported by Livingston et al. (12), who developed a selective immunoadsorbent for the DNA polymerase from murine leukemia virus. Gerwin and Milstein (6) have reported that columns of $(dT)_{12-18}$-cellulose provide a one-step enrichment procedure for the purification of DNA polymerase from Rauscher murine leukemia virus and from RD-114-infected cells. Due to the small amounts of protein used in each of these cases, purity of the isolated DNA polymerases could only be determined through indirect criteria, such as the absence of detectable nuclease activities (6, 12). The ability of an insolubilized DNA primer molecule such as oligo(dT) (as $(dT)_{12-18}$) to serve as an affinity matrix for the specific purification of RNA-dependent DNA polymerase from oncornaviruses and oncornavirus-infected cells seems surprising in view of the avidity with which cellular DNA polymerases also use such primers to catalyze DNA-dependent DNA synthesis (7, 21) and the selectivity of this insolubilized primer has recently been questioned by Sarin and Gallo (18). Thus far, the only template-primer which has been shown to be specific for RNA-dependent DNA polymerase utilization is poly(rC)·$(dG)_{12-18}$ (7, 17). Due to the apparent inability of cellular DNA-dependent DNA polymerases to utilize the synthetic template-primer poly(rC)·$(dG)_{12-18}$ (11, 17) and the well-known preference of RNA-dependent DNA polymerases to copy ribohomopolymeric templates over deoxyribohomopolymeric templates, (7, 17), we have investigated the use of insolubilized poly(rC) as an affinity matrix. In this report, we describe the use of poly(rC) derivatized to agarose as an affinity matrix for the rapid, high-yield purification of the RNA-dependent DNA polymerase from avian myeloblastosis virus (AMV).

MATERIALS AND METHODS

Virus isolation and disruption. AMV was supplied as plasma from infected chickens by Life Sciences, Incorporated, St. Petersburg, Fla., prepared under contract NO1CP33291, Virus Cancer Program, National Cancer Institute. Plasma was stored at −70 C and thawed just prior to use. All operations were performed at 0 to 4 C. After thawing, the plasma was filtered through four layers of gauze, layered atop a glycerol cushion, and virus was pelleted onto the cushion by centrifugation at top speed for 1 h in the SW 27 rotor. The virus was collected from the cushion, diluted threefold with buffer containing 0.01 M Tris-hydrochloride, pH 7.8, 0.15 M NaCl, and 0.01 M EDTA (TNE buffer), and layered over linear 20 to 50% (wt/vol) sucrose gradients prepared in TNE buffer. AMV was isopycnically banded at 105,000 × *g* in the SW 27 rotor for 15 h at 4 C, after which time the virus band was collected and used immediately.

AMV purified from plasma was pelleted at 150,000 × *g* at 4 C for 30 min. The viral pellet was suspended in disruption buffer consisting of TNE buffer containing 10% (vol/vol) Shell nonionic detergent p-40 (NP-40), 1% (wt/vol) sodium deoxycholate, 0.4 M

KCl, and 20% glycerol. Approximately 1 ml of disruption buffer was used for 2 mg of viral protein. Protein concentrations were determined by using the method of Lowry et al. (13) with bovine serum albumin (fraction V) as a standard. After complete mixing of the viral pellet with disruption buffer, the solution was kept at 0 C for 12 to 15 h. Remaining particulate debris was removed by centrifugation at 15,000 × *g* for 15 min at 4 C.

Preparation and properties of poly(rC)-agarose. Polycytidylic acid (2.5 μmol of polynucleotide per mg nominal weight) was purchased from Schwarz Bioresearch and 4.1 mg (55 A_{260} units) dissolved in 10 ml of 0.05 M potassium phosphate buffer, pH 8.0. Coupling of poly(rC) to Sepharose 4B (Pharmacia, a commercially available form of beaded agarose), was carried out by using the method of Poonian et al. (16). A 10-ml amount of settled Sepharose 4B was mixed with an equal volume of distilled water, cooled to 10 C in an ice bath, and the pH of the suspension was adjusted to 11 by using 6 M NaOH. Two grams of finely divided cyanogen bromide (200 mg per ml of settled agarose) were added, while stirring, and the pH of the mixture was maintained at 11 by manual titration with 6 M NaOH. After cessation of base uptake (about 20 min after addition of cyanogen bromide), the gel was rapidly washed by vacuum filtration with 15 to 20 volumes of cold 0.05 M potassium phosphate buffer, pH 8.0, and the moist, activated gel was rapidly mixed with the previously prepared poly(rC) solution. The activated agarose-poly(rC) mixture was gently stirred at 4 C for 12 to 16 h and the gel was then allowed to settle. A portion of the solution above the settled gel was centrifuged at 2,000 × *g* for 10 min to remove fine particles and the absorbance at 260 nm was measured. Over 95% of the input A_{260} units were bound to the activated agarose. The poly(rC)-agarose derivative was then washed with 200 volumes of distilled water followed by 200 volumes of 0.05 M potassium phosphate buffer, pH 8, after which no detectable absorbance at 260 nm could be found in the wash buffer.

The UV absorbance spectra of derivatized agarose were measured by a modification of the method of Barry and O'Carra (1), by using a Beckman Acta CV spectrophotometer in the double-beam mode of operation. To obtain spectra of agarose derivatives, samples were suspended in 50% (wt/vol) sucrose solution prepared in 0.05 M Tris-hydrochloride, pH 7.8. By this procedure, the agarose beads remained in uniform suspension for at least 5 min. The results of UV spectral scans performed in this manner are shown in Fig. 1. Poly(rC) in aqueous solution as well as poly(rC)-agarose displayed absorbance maxima at 272 nm, with poly(rC)-agarose showing an additional absorbance peak at 217 to 219 nm. The reference for spectral scans of poly(rC)-agarose consisted of an equal volume of unsubstituted agarose. UV spectral scans of aminoheptamethylimino-agarose prepared by standard procedures (14) also displayed an absorbance maximum at 217 to 219 nm (Fig. 1), suggesting that such an absorbance peak is due to the formation of imidocarbonate or isourea upon cyanogen bromide activation of agarose and not to alterations in the structure of poly(rC) caused by the coupling procedure.

After washing, the poly(rC)-agarose derivative was stored in 0.05 M potassium phosphate buffer, pH 8.0, at 4 C. Pasteur pipette columns of poly(rC)-agarose were poured just prior to beginning experiments, using glass wool as a bottom support.

Enzymes. *Escherichia coli* DNA polymerase I was kindly provided as a homogeneous preparation by Lawrence Loeb. The large fragment of *E. coli* DNA polymerase I produced by subtilisin cleavage of the polypeptide chain (20) was purchased from Boehringer-Mannheim, Inc. and used without further purification.

Template-primers. The template-primer nucleic acids used in the DNA polymerase assays were obtained from P-L Biochemical Laboratories. Polyadenylic acid (poly rA) and oligodeoxythymidylic acid $(dT)_{10}$ were annealed before use at an equimolar ratio of nucleotides by heating a solution (300 μmol of nucleotide in 0.05 M Tris-hydrochloride, pH 7.8) to 55 C for 5 min and then allowing the solution to cool slowly to room temperature. Polycytidylic acid was annealed to oligodeoxyguanylic acid $(dG)_{12\text{-}18}$ at a molar ratio of nucleotides of 10:1.5, respectively. Annealing was carried out at 80 C for 5 min after which the poly(rC)·$(dG)_{12\text{-}18}$ was allowed to cool slowly to room temperature.

Labeled compounds. Tritiated deoxynucleoside triphosphates were purchased from Schwarz-Mann. The specific activities of [^{3}H]dTTP and [^{3}H]dGTP as purchased were 16 and 15.6 Ci/mmol, respectively.

DNA polymerase assays. Reactions were carried out in a total volume of 0.1 ml and consisted of 50 mM Tris-hydrochloride, pH 7.8, 10 mM 2-mercaptoethanol, 25 μg of bovine serum albumin, 5 mM $MgCl_2$, and 80 μM [^{3}H]dTTP or [^{3}H]dGTP containing 0.5 μg of template. Resulting specific activities of deoxynucleoside triphosphates were 60 counts per min per pmol of dTTP and 50 counts per min per pmol of dGTP. Reactions were initiated by the addition of

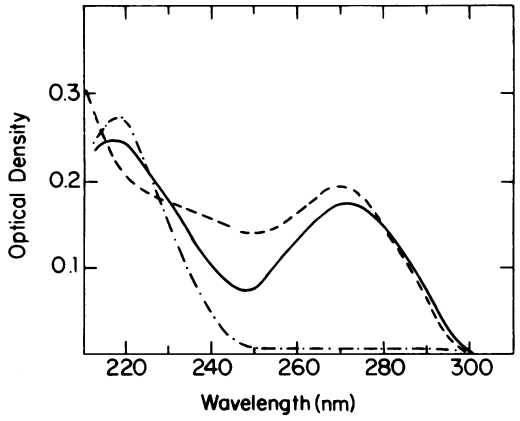

FIG. 1. *UV absorbance spectra of soluble poly(rC)* (-----), *poly(rC)-agarose* (——), *and aminoheptamethylimino-agarose* (-·-·-). *Spectra were determined as described in Materials and Methods.*

reaction mixture to enzyme fractions and were incubated at 37 C for 10 min. Reactions were terminated by the addition of 5% (wt/vol) trichloroacetic acid solution containing 0.01 M sodium pyrophosphate. Acid-insoluble material was collected by vacuum filtration onto Whatman glass fiber filters (GF/B). After drying, the filters were placed in toluene-based scintillation fluid and counted in the Beckman LS-250 liquid scintillation counter.

RNase H assays. The presence of RNase activity specific for the RNA strand of an RNA-DNA hybrid (RNase H) was assayed by a modification of the method of Grandgenett et al. (8). Reactions were carried out in a final volume of 0.25 ml consisting of 20 mM Tris-hydrochloride, pH 7.8, 5 mM 2-mercaptoethanol, 100 mM KCl, 2 mM $MgCl_2$, 0.1% (wt/vol) bovine serum albumin, and poly [^{14}C](rA)·poly (dT) (50 pmol of nucleotide). Reactions were initiated by the addition of enzyme fraction and were incubated at 37 C for 30 min. Reactions were terminated by addition of trichloroacetic acid and acid-insoluble counts were determined as in the DNA polymerase assays.

Molecular weight estimations: glycerol gradient. Velocity sedimentation was performed in preformed 10 to 30% (vol/vol) linear glycerol gradients in 0.01 M potassium phosphate buffer, pH 8.0, containing 1 mM dithiothreitol and 0.4 M KCl. AMV polymerase from peak column fractions was diluted 10-fold in the same buffer used to prepare the glycerol gradients and layered over a 5-ml gradient which was then centrifuged at 45,000 rpm for 16 h at 4 C using the SW 50.1 rotor. Fractions were collected from the top of the gradient using a Buchler Auto Densi-Flo and were assayed to determine the DNA polymerase activity profile. Parallel gradients were run using *E. coli* DNA polymerase I and the subtilisin-produced 70,000-molecular weight fragment of polymerase I to provide molecular weight markers. The marker gradients were assayed for DNA polymerase activity with poly(dA)·$(dT)_{10}$ as template-primer as previously described (15).

Disc gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed using the discontinuous Tris-glycine buffer system of Laemmli (10). Bovine serum albumin and ovalbumin were used as molecular weight standards. Fractions from each step in the purification of AMV DNA polymerase were diluted with an equal volume of 10% (wt/vol) trichloroacetic acid and allowed to sit at 0 C for 15 min. Precipitated protein was concentrated by centrifugation at 15,000 × *g* at 0 C for 10 min. The trichloroacetic acid was thoroughly drained from the pelleted protein after which the protein was dissolved in polyacrylamide sample buffer containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol in 0.01 M Tris-hydrochloride, pH 7.8 (50 to 100 μliters final volume of samples). Gel-fixing and destaining solution consisted of 454 ml of 50% methanol and 46 ml of glacial acetic acid. Protein bands were stained in a solution consisting of 454 ml of 50% methanol and 46 ml of glacial acetic acid in which 1.25 g of Coomassie brilliant blue R250 (Colab) had been dissolved. Staining was carried out for 2 h at 37 C. Optical density profiles of stained gels were obtained using the Joyce-Loebl Chromoscan apparatus and a slit width for maximum resolution.

RESULTS

Purification of AMV polymerase using rC-agarose. The purified, detergent-disrupted AMV was diluted 10-fold in buffer A consisting of 0.01 M potassium phosphate, pH 8.0, 1 mM DTT, and 10% (vol/vol) glycerol. The diluted, disrupted virus was passed through a 1- to 1.2-ml bed volume column of poly(rC)-agarose which had been previously equilibrated with buffer A. Flow rate was maintained at 5 ml per h. Figure 2 illustrates an experiment in which 4 mg of disrupted viral protein was passed through a poly(rC)-agarose column. From Fig. 2A it can be seen that almost all of the input protein was recovered in the effluent fractions while less than 1% of input DNA polymerase activity could be detected in the effluent. When protein could no longer be detected in the effluent fractions, a 0- to 0.5-M linear KCl gradient in buffer A (70 ml total volume) was applied to the column. All of the bound DNA polymerase activity could be recovered in this manner, with the peak of activity eluting at 0.2 M KCl. In this experiment, protein concentration in the eluate fractions could not be determined due to the low concentration of input protein. In addition to the poly(rA)·$(dT)_{10}$-directed DNA polymerizing activity (Fig. 2A), column fractions were assayed for poly(rC)·$(dG)_{12-18}$-directed DNA synthesis and RNase H activity as described in Materials and Methods (Fig. 2B). All activities were contained in the same fractions. As a control for poly(rC)-agarose, an identical column was prepared using unsubstituted agarose and examined for the possibility of artifactual binding by AMV DNA polymerase (inset to Fig. 2B). Less than 1% of input AMV DNA polymerase activity applied to the unsubstituted agarose column bound, although the quantity applied was far less than that which had been used for the poly(rC)-agarose column run (60 μg of disrupted viral protein), with the activity eluting at the breakthrough volume of the column. Addition of 0.4 M KCl to the unsubstituted agarose column removed the bound activity, suggesting that the small amount of artifactual binding observed may be due to slight charge attraction.

Table 1 illustrated the degree of purification obtained in an experiment in which 12 mg of viral protein was applied to a similar size poly(rC)-agarose column and eluted using a linear KCl gradient as in Fig. 2. The ability of small amounts of poly(rC)-agarose to bind large

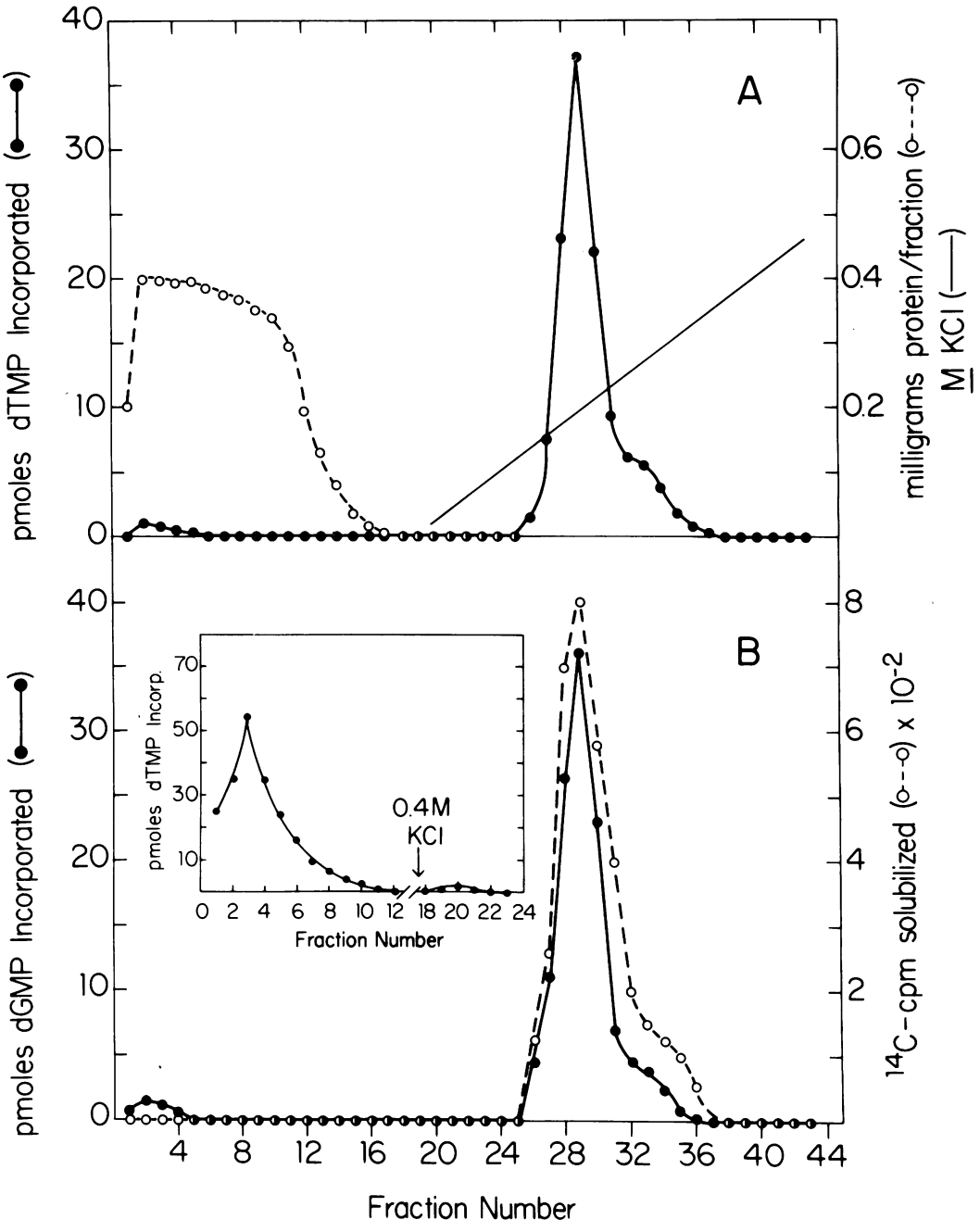

FIG. 2. *Chromatography of detergent-disrupted AMV on poly(rC)-agarose. Volume of initial effluent fractions = 1 ml. Volume of fractions collected after starting KCl gradient = 2.2 ml. KCl molarity is read on the same axis as protein concentration. Portions (5 μliters) of each fraction were assayed for (A) DNA polymerase activity with poly (rA)·(dT)$_{10}$. Protein concentration was determined as described in Materials and Methods. (B) DNA polymerase activity with poly (rC)·(dG)$_{12-18}$ and RNase H activity with [^{14}C]poly(rA)·poly (dT). Inset: chromatography of detergent-disrupted AMV on unsubstituted agarose. Fraction volume = 0.15 ml. Portions (10μliters) were assayed for DNA polymerase activity by using poly (rA)·(dT)$_{10}$.*

TABLE 1. *Purification of AMV DNA polymerase*

Protein source	Total protein (mg)	Total act[a]	Sp act[a]	Yield (%)
Soluble disrupted virus	12.3	4.3×10^6	360	100
Poly(rC)-agarose column effluent	12.0	0.03×10^6	2.5	
Pooled eluate column fractions	0.3	4.1×10^6	13,600	96

[a] Total activity is expressed as total units. One unit is defined as the amount of enzyme catalyzing the incorporation of 1 picomole of [^{3}H]dTMP into acid-insoluble material in poly(rA)·$(dT)_{10}$-directed DNA synthesis in 30 min at 37 C. Specific activity is expressed as units per microgram of protein.

quantities of AMV polymerase may be due to the high concentration of poly(rC) linked to the gel (approximately 400 μg per ml of settled agarose). The degree of purification of the pooled peak of eluted enzyme compares favorably with that obtained by Kacian et al. (9) using classical methods of enzyme isolation. Poly(rC)-agarose column fractions containing eluted enzyme were found to be free of DNA or RNA exonucleolytic activities. The extremely high yield of enzyme activity obtained using the poly(rC)-agarose column is consistent with the results previously described for affinity chromatography of various enzymes (5).

Determinations of molecular weight and purity of isolated DNA polymerase. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out on the disrupted virus preparation, the poly(rC)-agarose column effluent, and the pooled peak DNA polymerase activity eluate (Fig. 3). The optical density profiles of scanned gels reveal that virtually all of the viral protein except for bands corresponding to the α and β subunits of AMV DNA polymerase (8) are contained in the initial effluent fractions. Two major protein bands are observed in the gel containing peak polymerase activity fractions with molecular weights of 68,000 and 105,000. These molecular weights correspond to those previously described for the protein subunits of AMV polymerase (8, 9). Although polymerase activity of these subunits has not been measured directly, it appears most probable that they do indeed represent the enzyme.

Upon elution of AMV DNA polymerase activity from the poly(rC)-agarose column using a single concentration of KCl (0.4 M) rather than a linear gradient of increasing salt concentration, the AMV DNA polymerase peak is found to be contaminated with small quantities of a 27,000-molecular weight protein, which may correspond to the P27 protein previously described as a major component of the virion

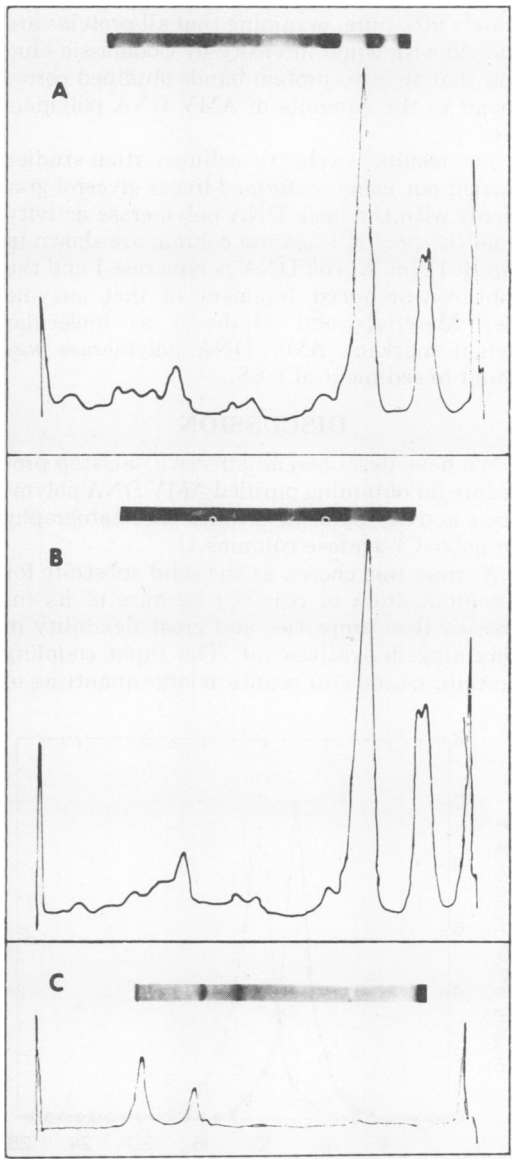

FIG. 3. *Sodium dodecyl sulfate-polyacrylamide gels and accompanying scans of those gels of (A) 100 μg of crude detergent-disrupted AMV viral protein; (B) 100 μg of poly(rC)-agarose column effluent protein; (C) 10 μg of protein from poly(rC)-agarose column eluate fraction containing peak DNA polymerase activity. The dark band seen at the right of gel C is due to the presence of 50 μg of egg white lysozyme used as a carrier. Direction of protein migration in all gels is from left to right.*

(data not shown) (22). Planimetric measurements performed on gel scans of fractions containing peak enzyme obtained by using linear gradients of increasing KCl concentration indicate that the eluted polymerase is approximately 90% pure, assuming that all proteins are stained with equal intensity by Coomassie blue and that the two protein bands obtained correspond to the subunits of AMV DNA polymerase.

The results of velocity sedimentation studies carried out using preformed linear glycerol gradients with the peak DNA polymerase activity from the poly(rC)-agarose column are shown in Fig. 4. Using *E. coli* DNA polymerase I and the subtilisin-produced fragment of that enzyme (see Materials and Methods) as molecular weight markers, AMV DNA polymerase was found to sediment at 6.6*S*.

DISCUSSION

We have described an effective one-step procedure for obtaining purified AMV DNA polymerase activity by using affinity chromatography on poly(rC)-agarose columns.

Agarose was chosen as the solid substrate for insolubilization of poly(rC) because of its increased flow properties and great flexibility in obtaining derivatives (5). The rapid coupling procedure used (16) results in large quantities of poly(rC) covalently linked to small amounts of agarose, thus eliminating the necessity of using large columns for purification, which might increase the possibility of artifactual binding of contaminating proteins. A further advantage of the use of the agarose in preparing polynucleotide columns is the ease with which the spectral properties of derivatives can be determined (see Materials and Methods). Through such studies it was determined that the coupling procedure used did not alter the poly(rC) with respect to its absorbance spectrum.

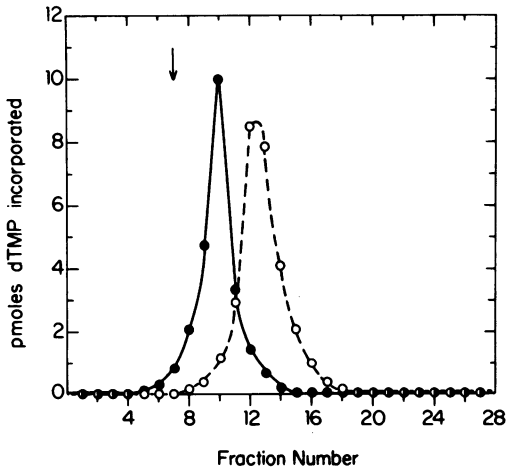

FIG. 4. *Glycerol gradient centrifugation of purified AMV DNA polymerase. Centrifugation was carried out as described in Materials and Methods. A 0.4-μg amount of purified AMV polymerase was layered onto the gradient and activity located after running using poly(rA)·$(dT)_{10}$ (○). A parallel gradient was run using 0.01 μg of E. coli DNA polymerase I, which was located by using poly(dA)·$(dT)_{10}$ (●). The arrow indicates the position of the subtilisin-cleaved DNA polymerase I fragment.*

Although previous studies on the isolation of RNA-dependent DNA polymerase activity from RNA tumor viruses reported excellent purification, such reports could only be presented as catalytic purity due to the small amounts of protein recovered after purification (6, 12). The studies described in this paper are the first involving affinity chromatography in which physical measurements are used to establish the degree of purification obtained for such polymerases.

The relationship of AMV DNA polymerase subunit structure to function was determined by Grandgenett et al. (8), who followed the classical method of enzyme purification devised by Kacian et al. (9). Two peaks of DNA-polymerase activity were observed upon phosphocellulose chromatography, designated PCI and PCII for their order of elution. Peak PCI (comprising 10 to 15% of total activity) contained both DNA polymerase and RNase H activity, and upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was found to consist of one major subunit which was designated α. Phosphocellulose peak PCII also contained both enzyme activities, and consisted of two subunits termed α and β in order of decreasing mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The α subunits of both PCI and PCII possessed identical mobilities and were found to have similar antigenic determinants. Molecular weights of α and β were found to be 65,000 and 105,000, respectively (8), similar to results obtained by Kacian et al. (9). Therefore, although the majority of AMV DNA polymerase isolated by classical biochemical procedures appears to have a native molecular weight of approximately 160,000 to 180,000 (8, 9) and exists as the αβ complex, all enzyme activities may be found in the α subunit (8). The function of the β subunit has not yet been determined. Since purification by affinity chromatography involves the least degree of biochemical manipulation of enzymes, it is interesting to note that all the AMV polymerase isolated by affinity chromatography ap-

peared as a single peak of activity corresponding to the PCII enzyme described by Grandgenett et al. (8). No evidence of the single subunit α containing both RNase H and DNA polymerase activity could be detected (8). The possibility also exists that the PCI enzyme may be produced by dissociation of the native polymerase after chromatography on DEAE-cellulose. As both forms of AMV DNA polymerase appear to possess identical catalytic activity, the possibility that they may both elute in the single peak obtained from the poly(rC)-agarose column should not be excluded. This likelihood may be resolved by phosphocellulose chromatography of the polymerase obtained using affinity chromatography.

The two-column procedure for the isolation of AMV DNA polymerase described by Kacian et al. (9) has proven to be an excellent purification method using classical enzyme isolation procedures. Poly(rC)-agarose affinity chromatography of disrupted AMV provides a more rapid procedure resulting in much higher yields of purified enzyme. Using AMV as a model system, and owing to the similar abilities of other RNA tumor virus DNA polymerases to utilize poly(rC)·$(dG)_{12\text{-}18}$ as a template-primer for DNA synthesis (7, 17, 18), it is expected that affinity chromatography on poly(rC)-agarose will prove a useful method for the isolation of other polymerases. By preparing selective polynucleotide and polydeoxynucleotide-agarose derivatives, it may be possible to separate cellular from viral DNA polymerases rapidly and efficiently. We are currently investigating this possibility using human leukemic cells in an attempt to isolate the RNA-dependent DNA polymerase previously reported (2, 19).

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