Correction. In the article "RNA-Linked DNA Fragments In Vitro," by Sugino, A. & Okazaki, R., which appeared in the January 1973 issue of Proc. Nat. Acad. Sci. USA 70, 88–92, due to errors made in the Proceedings Office, the following corrections should be made.

On page 89, left-hand column,

\[
\begin{array}{c}
\text{---P} \\
| OH | OH | OH | H | H | H | H \\
|---|---|---|---|---|---|---
\end{array}
\]

Alkaline hydrolysis

should appear as:

\[
\begin{array}{c}
\text{---P} \\
| OH | OH | OH | H | H | H | H \\
|---|---|---|---|---|---|---
\end{array}
\]

\[
\begin{array}{c}
\text{N} \\
\text{N} \\
\text{---P} \\
| OH | OH | OH | H | H | H | H \\
|---|---|---|---|---|---|---
\end{array}
\]

On page 90, right-hand column (top),

\[
\begin{array}{c}
\text{Py} \\
| OH | OH | OH | OH | H | H | H | H \\
|---|---|---|---|---|---|---|---
\end{array}
\]

Pancreatic RNase

should appear as:

\[
\begin{array}{c}
\text{Py} \\
| OH | OH | OH | OH | H | H | H | H \\
|---|---|---|---|---|---|---|---
\end{array}
\]

\[
\begin{array}{c}
\text{---P} \\
| OH | OH | OH | H | H | H | H \\
|---|---|---|---|---|---|---|---
\end{array}
\]

Correction. In the article "A Single Subunit from Avian Myeloblastosis Virus with Both RNA-Directed DNA Polymerase and Ribonuclease H Activity," by Grandgenett, D. P., Gerard, G. F. & Green M., which appeared in the January 1973 issue of Proc. Nat. Acad. Sci. USA 70, 230–234, the following correction should be made. On page 230, column 2, line 8 from top (under Enzyme Assays), "50 µM NaCl" should read: 50 mM NaCl.

Correction. In the article "Ion-Diffusion Potentials and Electrical Rectification Across Lipid Membranes Activated by Excitation-Induced Material," by Kalkwarf, D. R., Frasco, D. L. & Brattain, W. H., which appeared in the December 1972 issue of Proc. Nat. Acad. Sci. USA 69, 3765–3768, page 3767, under Results and Correlations, column 1, line 13, the magnitude 5.0 ± 0.6 µF/cm² should read: 0.5 ± 0.06 µF/cm².
A Single Subunit from Avian Myeloblastosis Virus with Both RNA-Directed DNA Polymerase and Ribonuclease H Activity  
(RNA virus/polyacrylamide gel electrophoresis)  

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Communicated by Robert J. Huebner, November 7, 1972  

ABSTRACT Two structurally distinct forms of RNA-directed DNA polymerase from avian myeloblastosis virus were resolved by chromatography on phosphocellulose and purified. In addition to RNA-directed DNA polymerase activity, both enzymes had ribonuclease H (RNase H) activity, which degraded the RNA moiety of RNA-DNA hybrids. As determined by sodium dodecyl sulfate-polyacrylamide disc-gel electrophoresis, one form had two subunits, alpha (α) and beta (β), with molecular weights of 65,000 and 105,000, respectively. The other had a single subunit, α, with a molecular weight of 65,000. The sedimentation coefficients of αβ and α, determined by glycerol gradient centrifugation in 0.35 M KCl, were 7.8 S and 5.2 S, respectively. Both enzymes had similar antigenic determinants and could not be distinguished by a differential response to several different RNA and DNA templates. We suggest that α, which contains both RNA-directed DNA polymerase and RNase H activity, is derived by dissociation of αβ, the function of the β subunit is unknown.

The discovery of an enzyme activity closely associated with RNA-directed DNA polymerase from avian myeloblastosis virus (AMV) (1), ribonuclease H (RNase H), has provided stimulus for the structural identification of specific enzyme activities that may be involved in viral DNA synthesis. RNase H specifically degrades the RNA moiety of RNA-DNA hybrids and is present in 10 different RNA tumor viruses (2). A major and a minor peak of DNA polymerase activity have been noted in lysates of AMV after phosphocellulose chromatography (3). The structure (4) and properties (3, 4) of only the protein with the major enzyme activity have been studied. In this report we present results of studies on the structure and catalytic properties of the proteins containing both enzyme activities. Our results show that both RNase H and DNA polymerase activity reside on a single polypeptide subunit.

MATERIALS AND METHODS  

Virus Purification and Nucleic Acid Isolation. Avian myeloblastosis virus, BAI strain A, was purified as described (5) from frozen virus preparations generously supplied by Dr. J. W. Beard, Duke University. AMV 70S RNA (6), adenovirus type 2 and 7 DNA (7), and SV-40 Form 1 DNA (8) were isolated by published procedures.

Preparation of RNA-DNA Hybrids. Calf-thymus [3H]-RNA-DNA (2 nmol/ml of RNA nucleotides, 2800 cpm/pmol) and the synthetic homopolymer hybrid, [3H]poly(A)·poly(dT) (100 μM [3H]poly(A), 10 cpm/pmol; 400 μM poly(dT)), were prepared as described (2).

Abbreviations: AMV, avian myeloblastosis virus; SDS, sodium dodecyl sulfate.

Enzyme Assays. Poly(A)·oligo(dT)-directed DNA polymerase activity was assayed at 37° in the following reaction mixture (0.09 ml): 50 mM Tris·HCl (pH 8.3), 50 mM NaCl, 3 mM dithiothreitol, 5 mM MgCl₂, and 0.1 mM [3H]TTP (500 cpm/pmol) with oligo(dT)₁₂₋₁₈ and poly(A) at 10 μg/ml each. AMV 70S RNA-directed DNA polymerase activity was assayed in a reaction mixture (0.09 ml) containing 50 mM Tris·HCl (pH 8.3), 50 mM NaCl, 3 mM dithiothreitol, 5 mM MgCl₂, 1 mM MnCl₂, 0.1 mM each of dGTP, dCTP, and dATP, 3 μM [3H]TTP (1.6 × 10⁶ cpm/pmol), and 10 μg/ml of AMV 70S RNA. With calf-thymus DNA (30 μg/ml) as template, the reaction mixture was identical except that AMV 70S RNA and MnCl₂ were omitted. Reactions were terminated by addition of 10 μl of 0.2 M EDTA (pH 7.4). The amount of polymer product formed was determined by assay on Whatman DEAE-cellulose (DE-81) paper discs (9).

The reaction mixture (0.1 ml) for assay of RNase H activity (1) contained 30 mM Tris·HCl (pH 7.8), 5 mM dithiothreitol, 100 mM (NH₄)SO₄, 2 mM MnCl₂, and either calf-thymus [3H]RNA·DNA (1 to 2 × 10⁶ cpm) or [3H]poly(A)·poly(dT) (4 × 10⁴ cpm). The reaction was terminated after 10 min of incubation at 37° by addition of cold 10% trichloroacetic acid (90 μl) and calf-thymus DNA (30 μg) as carrier. Radioactivity of acid-soluble material in the supernatant was determined after centrifugation at 12,000 × g for 15 min.

The assay for single-stranded nucleic acid was identical to the assay for RNase H except that 3 μM [3H]poly(U) (35 cpm/pmol) was used as substrate.

DEAE-Cellulose and Phosphocellulose Chromatography. These and subsequent purification steps were done at 4° unless otherwise specified. Just before enzyme purification, purified virus containing 500 mg of protein was dialyzed against 10% glycerol–10 mM potassium phosphate (pH 8.0)–2 mM dithiothreitol–0.1 mM EDTA for 2–4 hr. This buffer was used throughout the purification with changes in the glycerol and potassium phosphate concentrations as indicated. The virus suspension was adjusted to a protein concentration of 1 mg/ml in the above buffer, lysed with Nonidet P-40 (NP-40) at a final concentration of 0.2%, stirred for 10 min at 25°, and centrifuged at 12,000 × g for 8 min to remove debris. Less than 1% of the total enzyme activity was in the pellet. The supernate was applied to a 2.5 × 25 cm DEAE-cellulose (Whatman DE-52) column equilibrated with phosphate buffer. The column was washed with three column volumes of phosphate buffer and developed with a 700-ml linear gradient of 0.01–0.5 M potassium phosphate. The salt concentration was determined conductimetrically. The fractions in the single peak containing both DNA poly-
merase and RNase H activity were pooled; protein was precipitated by addition of solid ammonium sulfate to give 60% saturation. The precipitate was collected by centrifugation, dissolved in a minimum volume of 0.2 M potassium phosphate buffer, and dialyzed overnight against the same buffer.

The dialyzed DEAE-cellulose eluate was diluted 10-fold with 10 mM phosphate buffer and adsorbed to a 1 × 25 cm phosphocellulose (Whatman P-11) column. After a wash with four column volumes of phosphate buffer, the column was developed with a 200-ml linear gradient of 0.01–0.5 M potassium phosphate. The peaks of DNA polymerase activity were pooled and concentrated by cyclic dialysis against 50 and 10% glycerol in 0.2 M phosphate buffer. Each cycle required 24 hr to complete and reduced the sample volume by 50–60%. The dialysis bag was tied after each cycle to maintain the reduced volume. The last dialysis was with 50% glycerol, and the enzyme was stored at −20°C.

**Glycerol Gradient Centrifugation.** Before centrifugation the enzyme preparations were either diluted or dialyzed for 2 hr with 10% glycerol in the appropriate high and low salt buffers described below. Enzyme preparations purified on phosphocellulose were centrifuged in 4.8-ml gradients of 20–40% glycerol containing 0.35 M KCl, 50 mM Tris-HCl (pH 8.0), 0.2% NP-40, 3 mM dithiothreitol, and 0.1 mM EDTA (high salt). In the low salt gradient, KCl was omitted. Centrifugation was for 24 hr at 2°C at 46,000 rpm in an SW50.1 rotor. Fractions of 0.2 ml were collected from the bottom of the tubes and assayed. The peak fractions were pooled and concentrated by cyclic dialysis as described above against 0.2 M KCl–50 mM Tris-HCl (pH 8.0)–3 mM dithiothreitol–0.1 mM EDTA in 10 and 50% glycerol.

**Nondissociating Polyacrylamide Disc-Gel Electrophoresis.** Nondissociating polyacrylamide gels were prepared and run by the procedure of Brown (10), except 4% acrylamide was used in the absence of calf-thymus DNA. Enzyme preparations were diluted before electrophoresis with 0.1 M potassium phosphate (pH 7.5)–50 mM glycine–15 mM dithiothreitol. Gels (0.6 × 6.0 cm) were run at 10 mA per gel at 4°C for 4–8 hr. The gels were cut into 2-mm slices and eluted with 0.2 ml of 0.1 M potassium phosphate (pH 7.5)–10 mM dithiothreitol–10% glycerol.

Gel fractions to be examined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis were further eluted by addition of SDS to a final concentration of 1% and incubation overnight at 25°C. The gel fragments were removed by centrifugation, and the protein in the supernatant was precipitated with cold 10% trichloroacetic acid. The precipitate was collected by centrifugation and dissolved in denaturing solution (see next section). After addition of bromophenol blue to 0.1%, the pH was adjusted to 7 by addition of NaOH (2 N).

**SDS–Polyacrylamide Disc-Gel Electrophoresis.** SDS–polyacrylamide gels were prepared and run by the procedure of Maizel (11). Electrophoresis was performed with 0.5 × 11 cm gels of 6% acrylamide at 8 mA per gel at 25°C for 6 hr. Proteins were denatured for 10 min at 100°C with 1% 2-mercaptoethanol, 1% SDS, 10 mM sodium phosphate (pH 7.1), and 10% glycerol (denaturing solution). Gels were stained with 0.05% Coomassie blue in methanol–acetic acid–water (5:1:5, v/v) for 2 hr at 37°C and were destained with 10% methanol in 7.5% acetic acid for 48 hr.

**Enzyme Inactivation by Antibody.** Gammaglobulin, purified from antisera prepared against purified AMV polymerase that contained two subunits with molecular weights of 110,000 and 69,000 (4), was a gift from Drs. K. Watson and S. Spiegelman. Phosphocellulose peak I (0.06 μg) or peak II (0.03 μg) was mixed with this gammaglobulin suspended in 10 mM Tris-HCl (pH 8.3), 5 mM MgCl₂, 0.15 M NaCl, 20% glycerol, and 50 μg of bovine serum albumin in 60 μl. After 5 min at 37°C, DNA synthesis was initiated by addition of 0.3 nmol of [3H]TTP (1.6 × 10⁶ cpm/mmol) and poly(A)-oligo(dT) (1:1, 1 μg of each) to give a final volume of 100 μl. The amount of DNA product formed in 30 min was assayed.

**RESULTS**

**Purification of AMV Enzymes by DEAE-Cellulose and Phosphocellulose Chromatography.** RNA-directed DNA polymerase, RNase H, and single-stranded ribonuclease activities were routinely followed throughout purification. All the detectable DNA polymerase and RNase H activities in detergent-lysed AMV adsorbed to a DEAE-cellulose column. A single coincident peak of DNA polymerase and RNase H activity eluted from DEAE-cellulose at 0.09 M potassium phosphate. Single-stranded nuclease activity eluted as a single peak at 0.08 M potassium phosphate. A 15- to 20-fold purification of DNA polymerase was achieved on DEAE-cellulose with recoveries of 30–50%. Precipitation of the protein in the peak of enzyme activity with ammonium sulfate gave an additional 4-fold purification with no loss in total activity.

Chromatography on phosphocellulose gave two peaks of DNA polymerase activity (Fig. 1A). The first phosphocellulose peak (peak PCI) represented 10–15% of the total

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**Fig. 1.** Chromatography of AMV enzymes, purified on DEAE-cellulose, on phosphocellulose. Aliquots (2 μl) of 2-ml fractions were assayed for: (A) DNA polymerase with poly(A)-oligo(dT) (Δ) and AMV 70S RNA (○); (B) RNase H with native calf-thymus hybrid (□) and denatured hybrid (▲); and (B) single-stranded nuclease with [3H]poly(U) (●).
Glycerol Gradient Centrifugation. The centrifugation of phosphocellulose peaks I and II in high salt (0.35 M KCl) glycerol gradients is illustrated in Fig. 2. Peaks PCI and PCII sedimented as single components with sedimentation coefficients (s_{20,w}) of 5.2 S and 7.8 S, respectively, based on a 4.3 S value for hemoglobin (14). Based on these S values, the molecular weights of peaks PCI and PCII are about 90,000 and 160,000, respectively, from the equation $s_{20} = \frac{MW}{MW_s} \times (s_{20}-1)$ (15) (hemoglobin standard = 66,000). For both peaks PCI and PCII, the profiles of DNA polymerase activity with poly(A)-oligo(dT) and AMV 70S RNA were coincident. RNase H cosedimented with DNA polymerase from both peaks PCI and PCII. Single-stranded nuclease activity sedimented as a single component at 4.3 S in both enzyme preparations (data not shown). The recoveries of DNA polymerase activity were 70–80% in the presence of 0.2% NP-40, but 10–20% in its absence.

The sedimentation properties and stability of peak PCI in glycerol gradients was not affected by ionic strength. Peak PCII, however, aggregated in the absence of KCl, and recovery of DNA polymerase activity after glycerol gradient centrifugation in the absence of KCl was only 10–15%.

Properties of Purified Peaks PCI and PCII. The response of peaks PCI and PCII purified on glycerol gradients to several RNA and DNA polymers is shown in Table 1. The ratio of the specific enzyme activities with all templates tested was essentially constant.

The DNA endo- and exonuclease activity in both enzyme preparations purified on phosphocellulose was determined. We assayed DNA endonuclease activity by exposing adenovirus type 2 and 7 [\(^{14}C\)]DNA or SV-40 Form I \([^{14}C]\)DNA to enzyme under conditions for DNA synthesis, and then examining the sedimentation profiles of the DNA in alkaline sucrose gradients (16). Incubation of adenovirus type 2 and 7 DNA or SV-40 Form I DNA for 2 hr with either peak PCI

![Figure 2](image-url)

**Table 1. Template specificities of enzyme peak PCI and peak PCII**

<table>
<thead>
<tr>
<th>Templates*</th>
<th>pmol ([^{3}H]TTP) incorporated/10 min per (\mu g) of protein</th>
<th>Activity ratio (PCI/PCI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(A)-oligo(dT)</td>
<td>42.0</td>
<td>155</td>
</tr>
<tr>
<td>Poly(A)-poly(dT)</td>
<td>46.0</td>
<td>144</td>
</tr>
<tr>
<td>AMV 70S RNA</td>
<td>0.44</td>
<td>1.40</td>
</tr>
<tr>
<td>Activated calf-thymus DNA†</td>
<td>6.20</td>
<td>19.5</td>
</tr>
<tr>
<td>Native calf-thymus DNA</td>
<td>2.20</td>
<td>7.10</td>
</tr>
<tr>
<td>Denatured calf-thymus DNA‡</td>
<td>3.20</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Various polymers were used to assay peak PCI and peak PCII purified on glycerol gradients. The polymer concentrations were: activated, native, or denatured calf-thymus DNA, 30 \(\mu g/ml\); AMV 70S RNA, 20 \(\mu g/ml\); poly(A), 21 \(\mu g/ml\); poly(dT), 30 \(\mu g/ml\); and oligo(dT)_{10-19}, 30 \(\mu g/ml\). Peak PCI was 0.6 and PCI at 0.3 \(\mu g/ml\). The time course of enzyme activity was linear with each template for at least 20 min.

† Calf-thymus DNA was activated by treatment with DNase I (23).

‡ Calf-thymus DNA was denatured by heating at 100°C for 10 min.
glycerol was (1) Coomassie or peaks for tive was method ratio of the 

bovine-serum determined acetamide (17) in electrophoresis had 

were bands contaminating of DNAs poly(dAT), SV40 the DNA during 

a a2. The molecular weight was similar antigenic determinants.

Both DNA Polymerase and RNase H Reside on Alpha: Purification on Nondissociating Polyacrylamide Gels and Analysis by SDS–Polyacrylamide Gel Electrophoresis. To definitely establish that α possesses both DNA polymerase and RNase H activity, peak PCI was subjected to further purification on nondissociating polyacrylamide gels under conditions where DNA polymerase and RNase H activities were stable and could be eluted from the gels. For comparison, peak PCII was also examined. Typical results are shown in Fig. 4. Peak PCI migrated 7.5 mm farther than peak PCII after 4.5 hr of electrophoresis. Doubling the time of electrophoresis doubled the separation achieved. Analysis of DNA polymerase and RNase H eluted from gels of peak PCI (Fig. 4A) and peak PCII (Fig. 4B) showed that for each, both enzyme activities comigrated. In addition, AMV 70S RNA-directed DNA polymerase activity comigrated with the above two activities in gels from both enzymes (data not shown).
Chromatography of peak PCII a second time on phosphocellulose gave both peaks PCI and PCII. The α subunits of α and αβ had identical molecular weights and common antigenic determinants. These results strongly indicate that the α subunits are identical, and suggest that the source of α is αβ. The possibility exists that α and αβ are unique to different virus types (18), but this seems unlikely because of the identity in molecular weight and antigenic relatedness of α in α and αβ.

DNA polymerase and RNase H activity in AMV are located on α in peak PCI, and probably on the α subunit of αβ. The biological function of β in peak PCII has not been determined. The origin of the β subunit in peak PCII is also open to question. It is interesting that only avian RNA tumor viruses appear to have the β subunit associated with RNA-directed DNA polymerase (2-4, 12, 19, 20). Perhaps αβ in AMV is analogous to purified Qβ replicase, which is composed of both a viral and several host-specific polypeptide subunits (21, 22).

We thank Drs. H. Raskas and R. Roeder for critical reading of the manuscript. This work was supported by Contract PH43-67-692 within the Special Virus-Cancer Program of the National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Md. M. G. is a Research Career Awardee of the National Institutes of Health (5K6-A1-4759), and D. P. G. is a postdoctoral fellow of the American Cancer Society.