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SUMMARY

DNA polymerase from avian myeloblastosis virus has been purified by a combination of column chromatography and gel filtration methods. The isolated enzyme sediments at approximately 6 S and consists of two subunits of molecular weights 110,000 and 69,000. It is free of RNA and DNA endonuclease activity. The enzyme possesses the RNA-, DNA-, and hybrid directed polymerase activities found in the virion.

INTRODUCTION

The discovery of a ribonuclease-sensitive DNA polymerase activity in oncogenic RNA viruses was quickly extended to a wide variety of oncornaviruses. It was further shown that the product DNA was complementary to the RNA of the virion used as the source of the enzyme preparation. These findings were promptly followed by experiments that established the existence in these viruses of polymerase activities that respond to double-stranded DNA and, with a very high efficiency, to synthetic homopolymeric duplexes composed of polyribonucleotides polydeoxyribonucleotides, and hybrid structures of the two. The size of the DNA product synthesized was generally much less than that of the template employed.

In addition to the DNA polymerase activities, evidence was also found for DNA endo- and exonucleases, ligase, and a nucleosidetriphosphate phosphotransferase in the virion.

Purification of the RNA dependent DNA polymerase is a necessary prerequisite to an unambiguous analysis of the reaction mechanism. Purity is also required to delineate the relation of this polymerase activity to the others observed with DNA and synthetic homopolymeric duplexes as templates. Finally, pure enzyme should permit a decision on whether the nuclease, ligase and phosphotransferase activities are collectively or individually inherent and necessary components of the DNA polymerase function.

We report here the purification and characterization of the DNA polymerase activity from avian myeloblastosis virus.

Abbreviations: AMV, avian myeloblastosis virus; BBOT, 2,5-bis-2-(5-tert.-butylbenzoxazolyl)thiophene.

MATERIALS

Whatman microgranular DEAE-cellulose, DE52, 1.0 mequiv/g dry weight, and phosphocellulose, P11, 7.4 mequiv/g dry weight were obtained from Reeve Angel. Sephadex G-200 and CM-Sephadex C-50 were purchased from Pharmacia Fine Chemicals. Clarkson Chemical Company provided hydroxylapatite. Unlabeled nucleoside phosphocellulose, PII, 7.4 mequiv/g dry weight were obtained from Reeve Angel. Sephadex G-200 and CM-Sephadex C-50 were purchased from Pharmacia Fine Chemicals. Miles Laboratories supplied polynucleotides and triphosphates and dithiothreitol came from P-L Biochemicals. Miles Laboratories supplied polynucleotides and Bio-Rad Laboratories. Nonidet P-40 was a product of Shell Chemical Co.

METHODS

(1) Purification of avian myeloblastosis virus

Avian myeloblastosis virus (AMV), BAI strain A, was obtained by methods previously described4 from the blood of chicks in the terminal stage of myeloblastic leukemia5 and from infected myeloblasts suspended in tissue culture. Virus from blood plasma was purified essentially as described by CARNECIE20 and from infected myeloblasts suspended in tissue culture. Virus from chickens in the terminal stage of myeloblastic leukemia was collected dropwise from the bottom of the tube and those containing the RNA component were pooled and alcohol precipitated.

(2) Isolation of AMV RNA

Purified virus from blood plasma was lysed by adding sodium dodecyl sulfate to 0.5%. The suspension was extracted twice with phenol-creosol solution (prepared according to KIRAY6 and equilibrated with Tris-NaCl-EDTA buffer) and the RNA was precipitated by addition of 0.1 vol. of 3 M NaCl and 2 vol. of 95% ethanol. After a second alcohol precipitation, the RNA was layered onto a 10-30% glycerol in 0.01 M Tris-HCl (pH 8.5), 0.15 M NaCl, 1 mM EDTA (Tris-NaCl-EDTA buffer) onto a 6-ml pad of glycerol. The virus was removed from the pad, suspended in the same buffer without glycerol, and stored at -70°C.

Myeloblastosis virus produced in tissue culture was supplied by Dr. J. W. Beard, Duke University. The culture fluid had been concentrated 50-fold by centrifugation and contained from 2.5 x 1012 to 5 x 1012 virus particles per ml. After centrifugation for 10 min, the virus was concentrated against a 6-ml pad of glycerol at 27 000 rev./min for 1 h in the Spinco SW 27 rotor. Further purification was as previously described6.

(3) Preparation of polynucleotide duplexes

Polynucleotide duplexes were formed by annealing equimolar amounts of two complementary homopolymers at concentrations of approximately 100 μg/ml each in 0.01 M Tris-HCl (pH 7.4), 0.2 M NaCl at room temperature for 15 min.

(4) Polyacrylamide gel electrophoresis of proteins

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed by a modification of the method of SHAFFER et al.27. Protein samples were precipitated with an equal volume of 10% trichloroacetic acid, allowed to stand at 0°C for 15 min, and centrifuged at 16 000 x g for 30 min. Recovery was greater than 95%.

(5) Protein determination

Protein was measured by the method of LOWRY et al.17 using crystalline bovine albumin (Fraction V) as standard.

(6) Polymerase assay

The assay mixture for homopolymer templated reactions (total volume 0.1 ml) contained the following in μmoles: Tris-HCl (pH 8.3), 5.0; MgCl2, 0.6; 0.02 each of the required labeled and unlabeled deoxynucleoside triphosphates; and double-stranded homopolymer template, 1.2 x 104 pmole polymer phosphate in each strand. Reactions were incubated at 37°C for 10 min and terminated by the addition of cold 5% trichloroacetic acid.

After 10 min, the acid-precipitable radioactivity was collected on nitrocellulose filters and counted in 0.4% 2.5-butylbenzoate/3% hydroxybenzylpyridine (BBOT) in toluene.

Assays using natural RNA and DNA templates were prepared identically except that they contained 0.02 μmoles each of three unlabeled deoxynucleoside triphosphates and 4 μmoles of the fourth labeled triphosphate. Templates were used at levels from 1 to 2 μg per 0.1 ml assay.

Specific activities of the 3H-labeled triphosphates were 35-50 counts/minute per pmole for homopolymer-templated reactions and 350-500 counts/minute pmole for those using natural RNA or DNA templates.

(7) Preparation of AMV DNA polymerase

The procedure is described for 60 mg of purified viral protein. Larger amounts have been handled successfully by scaling up the various steps proportionately. 12 ml of AMV (5 mg/ml in 0.01 M Tris-HCl (pH 8.5), 0.15 M NaCl, 1 mM EDTA) were mixed in order with 1.2 ml Nonidet P-40, 1.2 ml 10% sodium deoxy-
cholate, and 3.6 ml 4 M KCl until homogeneous. The mixture was kept at 0°C for 15 min and then was centrifuged at 16 000 x g for 10 min. The pellet was discarded and the supernatant diluted to 10 times its volume with 0.01 M potassium phosphate (pH 7.2), 2 mM dithiothreitol, 10% glycerol.

The solution was applied to a 1.2 cm x 11.0 cm column of DEAE-cellulose carefully equilibrated with the same buffer. The column was washed with 80 ml of 0.05 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 10% glycerol, and eluted with 40 ml of 0.3 M potassium phosphate (pH 7.2), 2 mM dithiothreitol, 10% glycerol. The flow rate was about 36 ml/h.

The peak activity fractions from the DEAE-cellulose column were pooled and diluted to 3 times their volume with 0.01 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 10% glycerol. The material was loaded onto a 0.9 cm x 8.0 cm column of CM-Sephadex C-50 previously equilibrated with the same buffer. The column was washed with 8 ml of 0.1 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 10% glycerol and eluted with 12 ml of 0.3 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 10% glycerol. A flow rate of 15 ml/h was maintained. The peak fractions were pooled, glycerol was added to 50%, and the enzyme stored at -20°C.

(8) Phosphocellulose column chromatography of AMV DNA polymerase

The peak fractions from a DEAE-cellulose column (about 5 mg protein) were pooled and diluted 6-fold with 0.01 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 10% glycerol and applied to a 0.9 cm x 9.0 cm column of phosphocellulose equilibrated with the same buffer. The column was eluted with a 150-ml gradient from 0.05 M potassium phosphate (pH 8.0) to 0.5 M potassium phosphate (pH 8.0) containing 2 mM dithiothreitol and 10% glycerol. A flow rate was maintained at about 0.4 ml/min and about 1.5-ml fractions were collected.

(9) Hydroxylapatite column chromatography of AMV DNA polymerase

The peak fractions from a phosphocellulose column were pooled and diluted 5-fold with 0.01 M potassium phosphate (pH 7.2), 2 mM dithiothreitol, 10% glycerol and loaded onto a 0.9 cm x 9.0 cm column of hydroxylapatite equilibrated with the same buffer. The column was eluted with a 150-ml gradient from 0.05 to 0.5 M potassium phosphate (pH 7.2) containing 2 mM dithiothreitol and 10% glycerol. The flow rate was maintained at about 0.2 ml/min and about 2.0-ml fractions were collected.

(10) DNA cellulose chromatography of AMV DNA polymerase

DNA cellulose was prepared essentially as described by ALBERTS AND HERRICK. Clean cellulose (Munktell 410) was washed several times with boiling ethanol and distilled water to remove remaining pyridine. It was then pre-cycled with base and acid (0.1 M NaOH, water, 0.01 M HCl) and washed to neutrality with water. The cellulose was then thoroughly dried, first in air and then by lyophilization. Calf thymus DNA was dissolved in 0.01 M Tris-HCl (pH 7.4), 1 mM EDTA at a concentration of 1 mg/ml. The DNA solution was poured into petri dishes and mixed with the cellulose to form a slurry (approximately 1 g cellulose to 3 ml DNA solution). The material was extensively air dried, ground to a powder, and lyophi-
virus particles with detergent (0.7 % deoxycholate; 7 % Nonidet P-40) and salt (0.8 M KCl) at 0°. Glycerol gradient analysis in 0.1 M potassium phosphate showed that over 95 % of the enzyme activity sedimented at 8.4 S or less after treatment. Assays were performed using poly (rA) - poly (rU), AMV 70-S RNA, and M. lysodeikticus DNA as templates. Specific activities and recoveries were determined with poly (rA) - poly (rU). Lower concentrations of detergent or salt, while capable of releasing the enzyme from the virion, left much of the activity attached to material that sedimented at higher s values. After release the enzyme showed an absolute requirement for added template.

The extract was centrifuged at low speed to remove a small amount of material that reduced the flow rate of the DEAE-cellulose column. The pellet contained a negligible amount (< 3 %) of the activity.

Chromatography of AMV DNA polymerase on DEAE-cellulose

The solubilized enzyme was diluted 10-fold to reduce the concentrations of salt and detergent and loaded onto a column of DEAE-cellulose. The column was then exhaustively washed with 0.05 M potassium phosphate buffer, which removes the detergents and much of the protein. Virtually all the activity was retained throughout loading and washing. The enzyme was then eluted with 0.3 M potassium phosphate buffer. About 5 % of the protein is recovered from the column, together with most of the activity.

Fig. 1 shows a DEAE-cellulose column assayed, respectively, with three different templates: the homopolymer duplex poly (rA) - poly (rU), 70-S AMV RNA, and M. lysodeikticus DNA. Each template, the activity is found generally to coincide with the protein peak. The slight displacement of activity observed with various templates appears to be due to their differential sensitivity to contaminating proteins and salts.

Generally, greater than 90 % of the starting activity is eluted with the 0.3 M potassium phosphate. The DEAE-cellulose column step yields about a 20-fold enrichment of the enzyme.

CM-Sephadex chromatography of AMV DNA polymerase

The peak activity fractions from the DEAE-cellulose column were pooled, diluted 3-fold with low salt buffer, and loaded onto a column of CM-Sephadex. The column was washed with 0.1 M potassium phosphate and eluted with 0.3 M potassium phosphate. All of the enzyme activity is retained by the column during loading and washing. Fig. 2 shows the profiles obtained by assaying the column with three different templates. About 0.5-1 % of the total protein is eluted with the 0.3 M buffer.

The degree of purification after CM-Sephadex chromatography and the amount of activity recovered have varied with different batches of virus, but highly reproducible results are obtained when the same starting material is used. In every case, the column effectively removes all the acidic protein contaminants as measured by polyacrylamide gel electrophoresis at pH 8.9. Preparations using different batches of virus produced in tissue culture have yielded enzyme of 30-60-fold higher specific activity than the crude extract. Variability in yield is probably due to the instability of the enzyme. The history of the virus preparation may be of paramount importance in obtaining high yields and specific activities. It appears that certain treatments (e.g. multiple freezing and thawing) adversely affect the stability of the enzyme.

An actual enzyme preparation carried through the CM-Sephadex step is summarized in Table 1.

The CM-Sephadex enzyme was assayed as described in METHODS for RNA and DNA endonuclease activities. As can be seen in Figs. 3 and 4, no detectable break-
down of the nucleic acids occurred when incubated with purified enzyme under standard conditions, minus the deoxyriboside triphosphates.

Enzyme stored in 50% glycerol at -20°C has retained greater than 90% of its activity for more than 5 weeks.

**TABLE I**

PURIFICATION OF DNA POLYMERASE FROM AVIAN MYELOBLASTOSIS VIRUS

Assayed in the standard assay mixture using poly (rA) - poly (rU) as template. Specific activity expressed as pmoles dTMP incorporated per 10 min per µg of protein.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (µg)</th>
<th>Specific activity (units/µg)</th>
<th>Total activity (units)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Solubilized virus</td>
<td>155.4</td>
<td>26.4</td>
<td>4.1 x 10^6</td>
<td>100</td>
</tr>
<tr>
<td>(2) DEAE-cellulose column pool</td>
<td>8.0</td>
<td>487.5</td>
<td>3.0 x 10^6</td>
<td>95</td>
</tr>
<tr>
<td>(3) CM-Sephadex pool</td>
<td>1.5</td>
<td>909.0</td>
<td>1.4 x 10^6</td>
<td>34</td>
</tr>
</tbody>
</table>

**Evidence for two components in the AMV DNA polymerase**

Peak fractions from several columns were pooled, denatured with sodium dodecyl sulfate and mercaptoethanol and analyzed on 5% sodium dodecyl sulfate-polycrylamide gels. Fig. 5 shows the band patterns at various stages of purification. After DEAE cellulose chromatography there are still over 25 different polypeptide chains present. The second gel reveals that after CM-Sephadex two bands become very prominent suggesting that they may be responsible for enzyme activity. This is further supported by the third gel of material passed through a Sephadex G-200 column. Fig. 6 shows the activity profile from the Sephadex G-200 column and shows that the enzyme is separated from smaller ultraviolet-absorbing material.

It is of interest to note (Table II) that the Sephadex G-200 protein, which shows only two bands, retains the ability to respond to all the templates that were active with the crude detergent-disrupted virion preparations. Too much weight should not be given to the relative responses to the synthetic templates since they vary from one polymer preparation to another.
TABLE II
ACTIVITY OF PURIFIED AMV DNA POLYMERASE WITH VARIOUS TEMPLATES
Assays were performed as described in METHODS except that all reactions were incubated at 37oC for 10 min. Each contained 0.6 mg of Sephadex G-200 enzyme. [3H]dATP was used in all reactions except those with poly (dC) - poly (dG) and poly (rI) - poly (rC) where the label was in dGTP. Homopolymer duplex templated reactions contained 1.2 - 103 pmoles polymer phosphate in each strand. AMV RNA and M. lysodeikticus DNA were present at 1.2 mg and 2 mg per reaction, respectively.

<table>
<thead>
<tr>
<th>Template</th>
<th>Labeled base incorporated (pmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (dT) - poly (rA)</td>
<td>1294.6</td>
</tr>
<tr>
<td>AMV RNA</td>
<td>8.9</td>
</tr>
<tr>
<td>M. lysodeikticus DNA</td>
<td>7.6</td>
</tr>
<tr>
<td>Poly (dC) - poly (dG)</td>
<td>235.6</td>
</tr>
<tr>
<td>Poly (rA) - poly (rU)</td>
<td>440.5</td>
</tr>
<tr>
<td>Poly (rI) - poly (rC)</td>
<td>311.4</td>
</tr>
</tbody>
</table>

To gain further information on the association of the two principal bands with the polymerase activity, the behavior of the protein on other columns was examined.

Fig. 7. Phosphocellulose column chromatography of AMV DNA polymerase. AMV DNA polymerase (DEAE-cellulose fraction) was chromatographed on phosphocellulose as described in METHODS. 2 ml from each fraction were used to assay poly (dT) - poly (rA) templated activity and 20 ml from each for AMV RNA activity. A sodium dodecyl sulfate-polyacrylamide gel of the peak fraction is shown.

Fig. 8. Hydroxylapatite column chromatography of AMV DNA polymerase. AMV DNA polymerase (phosphocellulose fraction) was chromatographed on hydroxylapatite as described in METHODS. 10 μl from each fraction were used to assay poly [dT]·poly [rA] templated activity and 20 μl from each for AMV RNA activity. A sodium dodecyl sulfate-polyacrylamide gel of Fractions 26–37 is shown. Fractions 18–25 gave an identical but considerably lighter pattern.

Fig. 9. DNA cellulose chromatography of AMV DNA polymerase. AMV DNA polymerase (phosphocellulose fraction) was chromatographed on DNA cellulose as described in METHODS. 10 μl from each fraction were used to assay for activity with each of the indicated templates. A sodium dodecyl sulfate-polyacrylamide gel of fractions 29–32 inclusive is shown.


Fig. 10. Glycerol gradient centrifugation of AMV DNA polymerase. Centrifugation was performed as described in METHODS. Phosphocellulose enzyme was precipitated with an equal volume of saturated (NH₄)₂SO₄ (pH adjusted to 7.4 with NH₄OH) suspended in 0.1 ml of buffer and layered onto the gradient. The activity was located using poly (dC)·poly (rG) as the assay template and 10 μl from each fraction. The polymerase activity sedimented at approximately 6 S calculated with reference to bovine serum albumin (BSA, arrow) run on a parallel gradient.

Fig. 11. Sodium dodecyl sulfate-polyacrylamide gels of glycerol gradient peak. The peak fractions 20, 22, 24, 26, 28, 30 and 32 from the glycerol gradient shown in Fig. 10 were each precipitated with trichloroacetic acid and analyzed by electrophoresis in sodium dodecyl sulfate gels as described in METHODS. The gels show that the polypeptide chains sediment together and that they coincide with the activity peak. The amount of protein recovered on each gel corresponds to the relative activity of the respective fractions. The arrow indicates the activity peak.


Sedimentation of purified polymerase

The two components appear to behave as a single complex on a variety of columns. It was of obvious interest to see whether this held true in a sedimentation analysis. A phosphocellulose enzyme preparation was accordingly run on a glycerol gradient containing 0.2 M potassium phosphate as described in METHODS. Fig. 10 shows that the activity sediments as a sharp band with an s₂₀,w of 6 S corresponding
to a molecular weight of 110 000. It may be noted that a similar sedimentation value has been reported for the DNA polymerase of the Rous sarcoma virus. The peak fractions (20-32 inclusive) were precipitated and analyzed electrophoretically in sodium dodecyl sulfate-acrylamide gels. As may be seen from Fig. 11, the same two bands are observed as the principal components in the peak region. It is evident that they are sufficiently tightly complexed to behave as a single physical entity under these conditions.

**Molar ratios and molecular weights of the two components**

The molecular weights were determined on sodium dodecyl sulfate-acrylamide gels using polymers of ribonuclease A as molecular weight markers. As may be seen from Fig. 12, the two chains have molecular weights of 110 000 and 69 000.

If these two polypeptides are in fact subunits of the same enzyme, then the relative number of each present in an enzyme unit should be proportional to their

![Graph](image)

**Fig. 12. Determination of molecular weights of subunits.** The molecular weights of the polypeptide chains present in AMV DNA polymerase extracts were determined on sodium dodecyl sulfate-polycrylamide gels as described in Methods. The circles show the positions of polymers of ribonuclease A (mol. wt. 13 700) run as markers. The arrows mark the positions of the two major bands run on a parallel gel, indicating molecular weights of 110 000 and 69 000. Molecular weights of marker polymers range from 13 700 to 123 300.

![Graph](image)

**Fig. 13. Scan of sodium dodecyl sulfate-polycrylamide gel of CM-Sepahex enzyme.** Sodium dodecyl sulfate-polycrylamide gels were scanned, using a 0.05 mm slit, in the Gilford Model 2400 spectrophotometer equipped with linear transport. The pattern shows that the enzyme is approximately 90% pure assuming that all proteins are stained equally by Coomassie blue.


**TABLE III**

**WEIGHT RATIO OF CHAINS**

Gels were scanned as described by Berc~. The area under each peak was estimated by multiplying the peak height by the peak width at half-height.

<table>
<thead>
<tr>
<th>Gel No.</th>
<th>Enzyme preparation</th>
<th>110 000 mol. wt. polypeptide : 69 000 mol. wt. polypeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>DEAE-cellulose enzyme</td>
<td>1.3</td>
</tr>
<tr>
<td>53</td>
<td>CM-Sepahex enzyme</td>
<td>1.9</td>
</tr>
<tr>
<td>63</td>
<td>CM-Sepahex enzyme</td>
<td>1.7</td>
</tr>
<tr>
<td>64</td>
<td>Sepahex G-200 enzyme</td>
<td>1.5</td>
</tr>
<tr>
<td>67</td>
<td>CM-Sepahex enzyme</td>
<td>1.5</td>
</tr>
<tr>
<td>69</td>
<td>Sepahex G-200 enzyme</td>
<td>1.5</td>
</tr>
<tr>
<td>75</td>
<td>Phosphocellulose enzyme</td>
<td>1.4</td>
</tr>
<tr>
<td>83</td>
<td>Hydroxyapatite enzyme</td>
<td>1.3</td>
</tr>
<tr>
<td>88</td>
<td>Glycerol gradient enzyme</td>
<td>1.7</td>
</tr>
<tr>
<td>89</td>
<td>Glycerol gradient enzyme</td>
<td>1.6</td>
</tr>
<tr>
<td>90</td>
<td>Glycerol gradient enzyme</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>(Fraction 24, Fig. 11)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Fraction 26, Fig. 11)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Fraction 28, Fig. 11)</td>
<td></td>
</tr>
</tbody>
</table>

Average of 11 gels:

Expected value (assuming one of each polypeptide chain per enzyme molecule):

1.6

**TABLE IV**

**REQUIREMENTS OF AMV DNA POLYMERASE REACTION**

Complete system: 50 mM Tris-HCl, pH 8.3; 6 mM MgCl₂; 0.2 mM each dATP, dCTP, dGTP; 0.04 mM [³H]dTTP, 665 counts/min per pmole; 0.7 µg AMV DNA polymerase, phosphocellulose fraction; 2 µg AMV RNA; 0.4 mM dithiothreitol; 100 mM KCl; incubated 20 min at 37°.

<table>
<thead>
<tr>
<th>System</th>
<th>[³H]dTTP incorporated (pmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>12.4</td>
</tr>
<tr>
<td>-MgCl₂</td>
<td>0.0</td>
</tr>
<tr>
<td>-MgCl₂+0.6 mM MnCl₂</td>
<td>3.5</td>
</tr>
<tr>
<td>-dATP</td>
<td>0.4</td>
</tr>
<tr>
<td>-dCTP</td>
<td>0.3</td>
</tr>
<tr>
<td>-dGTP</td>
<td>0.5</td>
</tr>
<tr>
<td>-AMV RNA</td>
<td>0.1</td>
</tr>
<tr>
<td>-dithiothreitol</td>
<td>8.6</td>
</tr>
<tr>
<td>-KCl</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Properties and requirements of the reaction with purified AMV DNA polymerase

The available information on the optimal conditions and requirements of RNA-dependent DNA polymerases has thus far been derived from studies with detergent-disrupted virion preparations. Table IV summarizes a reexamination with a purified AMV polymerase. It will be noted that the reaction is dependent on RNA, the presence of all four deoxyriboside triphosphates, and a divalent cation. Mg²⁺ being superior to Mn²⁺ over a wide range of concentrations. The addition of dithiothreitol and KCl lead to marked improvement. Fig. 14 shows that the effect of the dithiothreitol is most readily apparent in the later stages of prolonged syntheses.

Fig. 14. Effect of dithiothreitol on AMV DNA polymerase activity. Reaction kinetics in the presence (●) and absence (○) of 0.4 mM dithiothreitol (DTT) are shown. The template is AMV 70-S RNA. The curves show that dithiothreitol is necessary for long-term synthesis. The exact time varies at which the reaction without dithiothreitol ceases.

DISCUSSION

The two-column procedure described for preparing purified DNA polymerase from avian myeloblastosis virus is a rapid and convenient procedure for obtaining reasonably pure enzyme in good yield. At the CM-Sephadex stage, enzyme from tissue culture virus is 90% pure or better. Substitution of phosphocellulose for CM-Sephadex chromatography gives similar results. If one starts with virus isolated from the plasma of infected birds, enzyme of even higher purity is obtained at corresponding stages of purification. Removal of residual contamination can be achieved by (NH₄)₂SO₄ concentration followed by either Sephadex G-200 or hydroxylapatite chromatography, or centrifugation through glycerol gradients.

Two polypeptide chains are found to be associated with the activity in a 1:1 ratio throughout purification by DEAE-cellulose, CM-Sephadex, Sephadex G-200, phosphocellulose, hydroxylapatite and DNA cellulose affinity chromatography as well as by glycerol gradient centrifugation. This suggests that they are subunits of the polymerase. However, proof that the two polypeptides are in fact subunits will require examination of the activities of the separated and reconstituted proteins. The possibility that the subunits may each have one or more of the activities associated with them individually cannot be ruled out at this time.

There is a 1.6-fold discrepancy between the molecular weight of the active form as determined by glycerol gradient centrifugation (110,000) and the sum of the molecular weights of the two polypeptide chains determined on sodium dodecyl sulfate-polyacrylamide gels (110,000 and 69,000). Assuming that the molecular weight determinations are not in error, the fact that the ratio of the chains is constant across the activity peak (Table III) in the glycerol gradient suggests that the discrepancy is due to differences in molecular asymmetry of the subunits as compared with assembled proteins. A quantitatively similar situation obtains with Qβ replicase in which the sum of the molecular weights of the subunits determined on sodium dodecyl sulfate gels is 1.7 times that determined by sedimentation for the native enzyme.

The value obtained for the sedimentation coefficient of the enzyme would seem to exclude the possibility that the molecule contains more than one of each subunit.

It is worth emphasizing that association of the two major polypeptides, even through an extensive series of purification steps, does not constitute proof that they represent the desired enzyme. As with all such enzyme purifications, minor contaminants (e.g. 1% or less) that might not be detectable could conceivably be responsible for the activity. We cannot prove rigorously at this time that the two principal polypeptide chains constantly observed in our active preparations compose the polymerase. However, in addition to their invariant presence and constant 1:1 ratio, several additional considerations argue for their being the enzyme components.

Throughout purification, there have been no minor bands that are consistently present in all preparations. The most frequently observed contaminant is a pair of bands representing approximately 4% of the total protein. From their gel mobilities, they have molecular weights of 190,000 and 210,000 and therefore would be expected to sediment much more rapidly, either individually or as a complex, than the polymerase. They are not enriched relative to the two major bands during the course of purification of the activity. It seems likely that they represent aggregates of the major bands due to the relatively large amount of protein applied to the gels.

Were the activity due to any contaminant present as a few percent of the protein, the specific activity of the purified enzyme would be many-fold greater than that observed. The most active preparations of E. coli DNA polymerase assayed with poly[d(A-T)], Qβ replicase assayed with Qβ RNA, and E. coli transcriptase assayed with poly[d(A-T)] and calf thymus DNA have specific activities, respectively, of 1.2, 3.2, 4.5, and 1.2 moles nucleoside monophosphate incorporated per sec per mol enzyme. The most active preparations of AMV DNA polymerase incorporate 1.2 moles dXMP per sec per mol enzyme. This value is in excellent agreement with those for the other purified polymerases and argues that the principal protein components are responsible for activity. These calculations encompass both initiation and chain elongation. They are not based on any assumptions concerning the numbers of enzyme molecules participating, but rather on the reported enzyme specific activities.

If in fact the enzyme is essentially pure, the amount of protein recovered should correlate with the number of enzyme molecules expected per virion. It is reasonable to assume that there is at least one polymerase molecule per virus particle. Since the virion contains 3.0·10⁹ daltons of protein and the molecular weight of the AMV DNA polymerase is 1.8·10⁹ daltons, one enzyme molecule per virion

* The highest value obtained for enzyme fractions described as homogenous was used. The reported specific activities were converted from arbitrary units to moles of nucleoside monophosphate per sec per mol of enzyme.
would represent 0.06% of the total protein. Normally, 0.3-1% of the starting protein is recovered, which would correspond to 5-17 enzyme molecules per virus particle. Were the enzyme a 2% contaminant of our preparation, each virion would contain only 0.1-0.3 polymerase molecule.

The availability of pure enzyme makes possible a re-examination of the nature of the intermediates and the final product uncomplicated by possibly irrelevant or interfering enzyme activities.

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