Isolation of the catalytic core of DNA polymerase alpha from rabbit bone marrow

Lee Pletts Goscin and John J. Byrnes

Veterans Administration Medical Center and The Center for Blood Diseases, University of Miami School of Medicine, Miami, FL 33125, USA

Received 21 May 1982; Revised and Accepted 1 September 1982

ABSTRACT

Modification of the purification procedures for rabbit bone marrow DNA polymerase [Byrnes, J. J., & Black, V. L. (1978) Biochemistry 17, 4226-4231] has increased the yield and stability of the enzyme thus allowing further purification. In particular, the higher molecular weight form, \( \alpha_1 \), has been more abundant. Additional purification has been obtained upon phosphocellulose and chromatofocusing column chromatography. SDS slab gel electrophoretic analyses of the eluates demonstrate a 135,000 molecular weight polypeptide in nearly pure form which correlates with DNA polymerase activity. Approximately 200,000 nmol of thymidine monophosphate is incorporated into DNA (mg of protein\(^{-1} \) h\(^{-1} \)) at 37°C. Similar to DNA polymerase alpha from other sources this enzyme is an acidic protein, is very sensitive to aphidicolin, and has no detectable 3' to 5' nuclease activity.

INTRODUCTION

DNA polymerase alpha was first identified more than 20 years ago. The purification and determination of its molecular structure has been complicated by a multiplicity of forms. Aggregation, proteolysis and possible association with other proteins of the replication complex are complicating factors. The DNA polymerase activity has been associated with a polypeptide in the range of 118,000-156,000 molecular weight by an increasing list of investigators (1-7), while others have described "alpha" type DNA polymerases apparently consisting of 40,000-70,000 molecular weight subunits (8-10). We previously reported the purification of DNA polymerase alpha from rabbit bone marrow to a specific activity of 30,000 U/mg protein (11). Two forms of alpha were evident by zone sedimentation and molecular sieve column chromatography studies. A higher molecular weight form, which eluted from Ultrogel first, was referred to as \( \alpha_1 \). It has a Stokes radius of 65 Å, a sedimentation coefficient of 85 and a calculated molecular weight of 215,000. The second form, \( \alpha_2 \) has a Stokes radius of 40.5 Å, a sedimentation coefficient of 65 and a calculated molecular weight of 100,000. Since that report we have made modifications in the procedure which have increased the
stability of the enzyme and have enhanced the recovery of the alpha\textsubscript{1} form. Furthermore, we have extended the purification. A polypeptide of 135,000 molecular weight having DNA polymerase activity has been identified and obtained in nearly pure form.

MATERIALS AND METHODS

Reagents

\(^{3}\text{H}\)-labeled deoxyribonucleoside triphosphates were purchased from New England Nuclear. Deoxyribonucleoside triphosphates, oligo dT, poly dA, and calf thymus DNA, were obtained from P&L Biochemicals. Activated calf thymus DNA was prepared as described (12). The protein dye reagent and all sodium dodecyl sulfate (SDS) electrophoresis reagents were from BioRad. Aphidicolin was obtained from Flow Laboratories, Bethesda, Md., and dissolved in dimethyl sulfoxide. All other chemicals were reagent grade.

DNA Polymerase Assay

The DNA polymerase reaction contained in a final volume of 0.1 mL: 40 mM morpholino propane sulfonic acid (MOPS) buffer, pH 6.75; 90 mM KCl; 0.4 mM MnCl\textsubscript{2}; 0.75 A\textsubscript{260} units of poly (dA); 1.88 x 10\textsuperscript{-2} A\textsubscript{260} units of oligo (dT); 19% glycerol; 10 \mu M \[^{3}\text{H}\]-dTTP (1000 Ci/mmol); 1 \mu g BSA and 1 to 20 \mu l of DNA polymerase. The reaction vessels were incubated 10 min at 37°C and stopped by addition of 0.1 mL of 0.1 M sodium pyrophosphate, 1.25 A\textsubscript{260} units of denatured salmon sperm DNA, and 4 mL of 5% cold trichloroacetic acid. The precipitate was collected on a Whatman (GF/C) filter and washed with 15 mL of cold 1% trichloroacetic acid and 5 mL of ethanol. The filter was dried and counted in 10 mL of toluene Liquifluor (New England Nuclear) in a Beckman LS-233 scintillation counter. One unit (U) of DNA polymerase catalyzes the incorporation of 1 nmol of deoxyribonucleoside monophosphate per hr at 37°C. When activated calf thymus DNA was used as template/primer, the reaction mixture contained 80 \mu g/mL activated calf thymus DNA and also 160 \mu M each of dATP, dGTP, dCTP and 10 \mu M[\(^{3}\text{H}\)]-dTTP (1000 Ci/mmol.) In either assay the counting efficiency was measured each time and ranged between 210 and 240 cpm/pmol of TMP.

SDS Slab Gel Electrophoresis

The 8% polyacrylamide (16 x 12 x 0.075 cm) slab gel system of Laemmli (13) and O’Farrel (14) was used. Forty \mu L samples were diluted two fold into buffer containing 2% SDS and 5% 2-mercaptoethanol and were heated for 30 sec at 100°C. Gels were run at 15 mA through the stacking gel and 20 mA through the lower gel. The gels were fixed in 50% methanol, 10% acetic acid for 45
min and were stained using the Oakley et al. (15) modification of the silver stain of Switzer et al. (16). Gels were photographed and traced using a Zeineh soft laser scanning densitometer with integrator. Protein in the bands was quantitated by comparison to known concentrations of myosin electrophoresed in the same gel. The silver stain was linear and directly proportional over a range of 2-100 ng of protein. The absolute quantity of protein may be somewhat more or less due to amino acid composition differences between DNA polymerase alpha and myosin. However, myosin was selected for a reference as it migrates near DNA polymerase alpha in the gel.

Other Procedures

3' to 5' exonuclease activity was assayed by measuring the release of [3H]-dTMP from poly(dA-dT)-[3H]-dTMP (17). Protein concentrations were measured using the Bradford method (18) with BSA as the standard. The pH and conductivity of solutions were measured with a Radiometer pH meter and a Yellowspring conductivity bridge.

Purification of DNA Polymerase Alpha

DNA polymerase was purified from erythroid hyperplastic rabbit bone marrow through Step VIII but with several important modifications of previously described procedures (11, 17). Briefly, rabbits were injected with phenylhydrazine to induce erythroid hyperplasia. The marrow obtained from the long bones of 50-54 rabbits yielded approximately 120 g of starting material. The cells were washed and lysed as described except that 0.2 mM phenylmethylsulfonyl fluoride (PMSF) from Calbiochem was included in the lysis buffer. After centrifugation of the cell lysate at 30,000g for 15 min, the supernatant was brought to 5 mM MgCl₂ and then centrifuged at 78,000g for 2 h. The resulting "microsomal" pellets were homogenized in 50 mL of 50 mM Tris-HCl, pH 7.8, 25% (v/v) glycerol, 0.1 mM EDTA, and 1.0 mM DTT (TGED buffer) containing 1.0 M KCl, and kept on ice overnight. The homogenate was centrifuged at 150,000g for 90 min, and the supernatant was retained. Ammonium sulfate was added to 60% saturation. The precipitate was collected by centrifugation at 30,000g for 15 min and resuspended in 25 mL of TGED buffer containing 0.5% poly (ethylene glycol) 6000 (PEG). All buffer solutions thereafter contained 0.5% PEG. Phosphocellulose, diethylaminoethyl-Sephadex, hydroxylapatite, a second phosphocellulose, and Ultrogel AcA 34 column chromatography were performed as previously described. All procedures were done at 4°C, and between procedures the material was stored at -70°C; whenever possible, plastic ware was used in contact with the enzyme.

Step IX. Further purification of the enzyme was obtained by chromatography
on a 3rd phosphocellulose column. Eight mL of enzyme, generally 800-1000 units, were diluted 3 fold in 25% glycerol, 0.5% PEG and 1 mM DTT (GPD) and applied to a 0.67 cm² x 1.5 cm phosphocellulose column which had been equilibrated with GPD. The column was washed with 6 mL of GPD containing 0.1 M ammonium sulfate and the enzyme was eluted by 1 mL stepwise application of GPD containing 0.125 M increments in ammonium sulfate. Fractions (0.5 mL) were collected and assayed for DNA polymerase and exonuclease and analyzed in SDS gels.

Chromatofocusing of DNA Polymerase Alpha  
A 1.5 cm² x 24 cm column of Pharmacia Polybuffer exchanger PBE94 was equilibrated with 0.025 M imidazole, pH 7.4, and 1 mM DTT. Five ml of polybuffer 74, pH 4.0 (Pharmacia), 15% glycerol, 0.5% PEG, 0.025 M imidazole and 1 mM DTT were added before enzyme was loaded. Enzyme from Step VII or VIII was desalted on a Sephadex G25 column eluted with 15% glycerol, 0.5% PEG, 1 mM DTT, and 0.025 M imidazole, pH 7.4, and then applied to the chromatofocusing column. A pH gradient was generated by passing 150 mL of polybuffer 74, pH 4.0, 15% glycerol, 0.5% PEG, 0.025 M imidazole, and 1 mM DTT through the column at 11 mL/min. Fractions were assayed for DNA polymerase and pH and examined in SDS gels.

RESULTS

A representative purification of DNA polymerase alpha is outlined in Table 1. In comparison to our previously reported result (11) the yield of enzymatic activity has been increased by the inclusion of PMSF and PEG in the enzyme buffers. Previously, 7500 U of DNA polymerase were obtained from similar starting material after Step III; since these changes generally close to 40,000 U are obtained. The amount of enzyme activity recovered in subsequent Steps IV thru VIII has increased proportionately, but the specific activity has been about the same as previously obtained.

The bimodal elution profile of DNA polymerase alpha upon molecular sieve chromatography (Step VIII) has been different since these changes. Previously the larger species, alpha1, eluted in a less defined pattern than the smaller form, alpha2, which eluted sharply and was the predominant form (11). Since these changes, the amount of alpha1 has been greatly increased and has eluted in a well defined pattern; the amount of alpha2 has remained about the same (Fig. 1). The specific activity of DNA polymerase alpha after Step VIII is generally about 35,000 U/mg protein and 1500-2200 U are usually obtained. Around 200 units of alpha2 are obtained with a specific activity about 12,000 U/mg protein. As previously reported, the elution of
TABLE I: PURIFICATION OF RABBIT BONE MARROW DNA POLYMERASE ALPHA

<table>
<thead>
<tr>
<th>STEP</th>
<th>U RECOVERED</th>
<th>U RECOVERED</th>
<th>SPECIFIC ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1978)</td>
<td>(1982)</td>
<td>(mg protein)^-1 h^-1</td>
</tr>
<tr>
<td>I</td>
<td>30,000 g supernatant</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>II</td>
<td>Extract of 180,000 g pellet</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III</td>
<td>Ammonium sulfate precipitation</td>
<td>7,600</td>
<td>41,650</td>
</tr>
<tr>
<td>IV</td>
<td>Phosphocellulose column chromatography</td>
<td>5,100/7,600</td>
<td>31,970/41,650</td>
</tr>
<tr>
<td>V</td>
<td>DEAE-Sephadex</td>
<td>2,125/5,100</td>
<td>20,540/31,970</td>
</tr>
<tr>
<td>VI</td>
<td>Hydroxylapatite column chromatography</td>
<td>965/2,125</td>
<td>15,600/20,540</td>
</tr>
<tr>
<td>VII</td>
<td>Second phosphocellulose column chromatography</td>
<td>683/965</td>
<td>6,800/7,900 a</td>
</tr>
<tr>
<td>VIII</td>
<td>Ultrogel AcA 34 column chromatography</td>
<td>400/450</td>
<td>2,097/2,652 b</td>
</tr>
<tr>
<td>IX</td>
<td>Third phosphocellulose column chromatography</td>
<td>ND</td>
<td>871/975 c</td>
</tr>
</tbody>
</table>

The yield of enzyme is lower than the efficiency of recovery for the following reasons:
(a) only DNA polymerase free of exonuclease activity from Step VI is used for further purification
(b) only 2 mL of the highest activity fractions from Step VII are used
(c) only the highest activity fractions from the center of the alpha1 eluate from Step VIII are used.

alpha1 corresponds to a Stokes radius of 65 Å. There is no detectable 3' to 5' exonuclease in association with either form of DNA polymerase alpha. The assay used readily detects the 3' to 5' exonuclease associated with DNA polymerase delta at a comparable stage of purification and activity (19).

Nonspecific loss of protein and unstable enzyme activity previously made it impossible to proceed beyond Step VIII. The addition of 0.5% PEG to the enzyme buffers has resulted in excellent recovery and stability of the activity in Steps VIII and IX. Consequently, further purification of alpha1 has been accomplished by chromatography on a small phosphocellulose column (Step IX). Only fractions with very high activity from the center of the alpha1 peak were used for further purification. Generally 800-1,000 U of DNA poly-
Figure 1. Ultrogel AcA 34 Column Chromatography (Step VIII). DNA polymerase alpha (2652U) was chromatographed on a 4.9 cm² x 57 cm Ultrogel column. Ten μL aliquots were assayed for DNA polymerase using poly(dA)/oligo(dT). Protein was measured by the Bradford method. The Stokes radius of alpha was determined using the following standard proteins to calibrate the column: ovalbumin (27.3 Å), BSA (35 Å), aldolase (45 Å) and ferritin (79 Å).

DNA polymerase were applied to the phosphocellulose column. The enzyme elutes as a small and quantitatively variable minor peak at 0.10M (NH₄)₂SO₄ and a major peak at 0.14M (NH₄)₂SO₄. The major peak usually contains between 50 and 75 percent of the applied activity. DNA polymerase activity on poly (dA)/oligo (dT) and activated calf thymus DNA are similar across the minor peak. Poly (dA)/oligo (dT) is about 3 times more active a template as activated calf thymus DNA across the major peak.

Electrophoretic analysis of the enzyme has been carried out. Aggregation of the enzyme to high molecular weight species under the conditions of nondenaturing electrophoresis limited the usefulness of those studies. Thus, we proceeded to SDS polyacrylamide gel analysis of the Step IX enzyme. This revealed only one polypeptide extending across the major peak of DNA polymerase activity. The concentration of this polypeptide correlates closely with the DNA polymerase activity (Fig. 2). Furthermore, in most fractions this polypeptide is nearly pure to visual inspection (Fig. 3). Analysis of the electrophoretic migration of this polypeptide by the method of Weber and Osborne (20) indicates an approximate molecular weight of 135,000 daltons (Fig. 4). The specific activity of the DNA polymerase based upon microdensi-
Figure 2. Third Phosphocellulose Column, Major Peak. Ten μL aliquots were assayed for DNA polymerase activity with poly (dA)/oligo (dT) (○○). The amount of 135,000 molecular weight polypeptide in 40 μL aliquots was determined by microdensitometry of the SDS gel and is represented by (△△); (NH₄)₂ SO₄ concentration (△△) was determined by conductivity measurement.

Figure 3. SDS Slab Gel Electrophoresis of Step IX, DNA Polymerase Alpha. Electrophoresis is from top to bottom. Twenty μL of peak fraction 22 are in lane 1; lane 2 contains 20 μL of enzyme buffer as a background control for the silver stain. The upper arrow indicates DNA polymerase alpha and the lower arrow shows the tracking dye. The alpha polypeptide band contains 28 ng of protein.
Figure 4. Determination of molecular weight of DNA polymerase alpha polypeptide. The following six standards and DNA polymerase alpha were electrophoresed on a SDS slab gel and stained with silver: myosin (200,000); DNA polymerase delta (122,000); phosphorylase b (92,500); BSA (66,000); ovalbumin (45,000); chymotrypsinogen (25,000). Their migration relative to the tracking dye is plotted versus log molecular weight.

ometric quantitation of the 135,000 molecular weight polypeptide is 200,000 U/mg protein on poly (dA)/oligo (dT).

DNA polymerase activity across the minor peak was obtained only in fractions containing the 135,000 molecular weight polypeptide. However, there are additional polypeptide bands between 50,000 and 70,000 molecular weight which also have an elution pattern closely related to the DNA polymerase activity. These polypeptides may contribute to the minor peak or may modify the polymerase activity presumably inherent in the 135,000 molecular weight polypeptide. They have been variable in relative amount and require further study for definition.

Chromatofocusing was explored as an additional or alternate purification step. Enzyme from Step VII or Step VIII was chromatofocused as described. DNA polymerase alpha elutes sharply at pH 5.2. Analysis of the polypeptide composition of the eluate by SDS gel electrophoresis also demonstrated strict correlation of the 135,000 dalton polypeptide with the DNA polymerase activity (Fig. 5). Four additional polypeptides with molecular weights of 66,000, 63,000, 56,000 and 54,000 daltons are also seen in the gel in a similar pattern to the DNA polymerase activity. Unfortunately, recovery of applied enzymatic activity has been disappointing, amounting to less than 25%. Furthermore, the specific activity based upon quantitation of the 135,000
Figure 5. Chromatofocusing of DNA polymerase alpha. Thirteen hundred U of Step VII DNA polymerase were chromatofocused on PBE94 polybuffer exchanger. Twenty µL aliquots were assayed on poly(dA)/oligo(dT) and pH was measured. Forty µL aliquots were examined in SDS gels and the amount of the 135,000 molecular weight polypeptide was determined by microdensitometry of the gel and is represented by (Δ-Δ).

Aphidicolin inhibits synthesis by the 135,000 molecular weight form of DNA polymerase alpha in an expected fashion. DNA synthesis on activated calf thymus DNA is inhibited 50% at 8 µg/mL when all required deoxyribonucleoside triphosphates are present. However, truncated DNA synthesis in the absence of any one of the required deoxyribonucleoside triphosphates is more sensitive to the effect of aphidicolin. The absence of dCTP especially enhances the inhibitory effect of aphidicolin and DNA synthesis is 90% inhibited at 8 µg/mL (Fig. 6). Synthesis on poly(dA)/oligo(dT) by the 135,000 molecular weight polypeptide is 50% inhibited at 4 µg/mL (not shown).

DISCUSSION
These studies of DNA polymerase alpha from rabbit bone marrow demonstrate a core polypeptide of 135,000 molecular weight. By itself the 135,000 molecular weight polypeptide is an active DNA polymerase with features typical of alpha type DNA polymerase: i.e., a molecular weight >100,000, no detectable 3' to 5' exonuclease, an acidic isoelectric point, and sensitivity to aphidicolin. Also, previous studies demonstrated this DNA polymerase is
Figure 6. Inhibition of DNA polymerase alpha by aphidicolin. The enzyme was assayed on activated calf thymus DNA with various concentrations of inhibitor (○—○). Truncated synthesis on calf thymus DNA in the absence of dCTP with various concentrations of aphidicolin is shown by (□—□). Without aphidicolin 105 pmol TMP was incorporated into DNA in the standard assay. When dCTP was omitted 21 pmol TMP was incorporated.

very sensitive to the sulfhydryl inhibitor N-ethylmaleimide (11).

Association with as yet poorly defined, smaller polypeptides may modify the behavior of the 135,000 molecular weight core. This association presumably gives rise to a higher molecular weight form evident on molecular sieve chromatography. The molecular weight of DNA polymerase alpha1 based upon Ultrogel elution corresponding to a Stokes radius of 65Å is 300,000. When corrected for its sedimentation behavior, the calculated molecular weight of alpha is 215,000 (11). Characterization of the interacting polypeptides and the functional modification of the DNA polymerase which they cause is an important aspect to pursue. This certainly will be facilitated by the isolation of the catalytic "core."

Several groups of investigators have described DNA polymerase alpha to consist of two or more polypeptides that fall in the size range between 40,000 to 70,000 daltons. Chen, et al. (10) presented evidence for a 54,0002 - 47,0002 subunit structure of purified murine myeloma alpha polymerase. Grummt, et al. (9) described calf thymus alpha polymerase as composed of seven distinct subunits between 64,000 and 52,000 daltons. Polymerase alpha from human KB cells was reported to be composed of 76,000 and 66,000 dalton subunits (8) but recently modified to be a quartet of 70,000, 65,000, 59,000 and 55,000 daltons (21). Clearly our findings are different from these. However, the alpha2 form has not been structurally
characterized. Alpha2 has a calculated molecular weight of 100,000 and could be composed of similar subunits.

In contrast, other investigators have described DNA polymerase alpha to consist of a large polypeptide component ranging from 118,000 to 156,000 daltons, together with several smaller polypeptides in the 40,000 to 70,000 dalton size range. Several groups have studied the calf thymus enzymes. Holmes, et al. (1) described multiple forms of calf thymus DNA polymerase alpha. They concluded that calf thymus DNA polymerase alpha is a 155,000 molecular weight polypeptide which associates with material of 50,000-70,000 molecular weight to give other forms. Grosse and Krauss (4) demonstrated polypeptides of 148,000, 59,000, 55,000 and 48,000 daltons associated with a form of calf thymus DNA polymerase alpha. The 148 kilodalton subunit appeared to carry the enzymatic activity. They have also reported 134 and 123 kilodalton subunits in another form of calf thymus alpha polymerase (22). Most recently, Albert, et al. (6) demonstrated a form of calf thymus DNA polymerase alpha consisting of an abundant 118,000 dalton polypeptide as well as five lower molecular weight polypeptides between 54,000 and 64,000 daltons. The 118,000 dalton polypeptide, was shown to be catalytically active. Peptide mapping indicated that the 118,000 dalton polypeptide shares extensive primary structure homology with 57,000, 58,000 and 64,000 dalton polypeptides and some limited homology with 54,000 and 56,000 dalton polypeptides. These results suggested a common precursor with a molecular weight of more than 140,000 daltons. Association of the calf thymus DNA polymerase alpha "core" polypeptide with the accessory subunits has been reported to result in differences in the utilization of synthetic template initiator complexes (23) and kinetic differences (24).

The DNA polymerase alpha of regenerating rat liver was purified to near homogeneity by Mechali, et al. and five polypeptides were resolved (2). The catalytic activity correlated with a 156,000 dalton polypeptide. The specific activity of the catalytic subunit was higher when associated with the other 54,000-64,000 dalton polypeptides.

Lamothe, et al. (5) described three forms of DNA polymerase alpha from Hela cells. A 140,000 dalton polymerase core polypeptide was common to the subunit composition of the three forms. However, additional polypeptide components in the 69,000 to 24,500 dalton range were responsible for structural and functional distinction. In particular two subunits of 47,000 and 24,500 daltons enhanced the polymerase activity on DNA template primers that contained extended single stranded regions.
Banks, et al. (3) demonstrated that DNA polymerase alpha type from Drosophila melanogaster embryos is composed of four polypeptides with molecular weights of 43,000, 46,000, 58,000 and 148,000 daltons. Villani, et al. (25) reported that the 148,000 subunit is required for DNA polymerase activity and the four polypeptides are structurally distinct from one another.

Yamauchi, et al. (7) purified DNA polymerase alpha from chick embryo extract. Two clusters of polypeptide bands were seen in SDS gels of the apparently pure enzyme. One was composed of 3-4 polypeptides between 130,000-155,000 molecular weight. The other consisted of four distinct polypeptides corresponding to 59,000, 56,000, 54,000 and 51,000 molecular weight. Tryptic peptide analysis indicated that the structures of all four 51,000 - 59,000 polypeptides were very similar. In addition, polypeptides in the 145,000 - 155,000 region had almost identical structure with those in the 130,000 - 140,000 region. No structural homology was observed between the high molecular weight polypeptides and the low molecular weight polypeptides.

Also evidence from renaturation of enzymes which have been electrophoresed in SDS polyacrylamide gels indicates that DNA polymerase activity from several mammalian tissues including calf thymus resides in a relatively large polypeptide, about 125,000 daltons (26). Our findings are consistent with these reports. In spite of the heterogeneity of results obtained in one tissue (calf thymus), it seems that progress and some consistency in the determination of the structure of eukaryotic DNA polymerase alpha is occurring. A high molecular weight, core polypeptide has been described from four mammalian tissues: calf thymus, rat liver, Hela cells and now, rabbit bone marrow. However, this is the first isolation of the "core" polypeptide without accompanying smaller polypeptides and in highly active form.

ACKNOWLEDGEMENTS: We appreciate the excellent technical assistance of Ms. Teresa Ferrer and Ms. Maria Serrano. This work was sponsored by the Veterans Administration. L.P.G. was supported by training grant #5 T32 AM07114 from the Institute of Arthritis, Metabolism and Digestive Diseases of the N.I.H.

REFERENCES

6034